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Feline Upper Respiratory Disease Complex: The detection and epidemiology of respiratory pathogens in Midwestern feline shelter populations

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Feline Upper Respiratory Disease Complex: The detection and epidemiology of respiratory pathogens in Midwestern feline shelter populations

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ABSTRACT

Feline Upper Respiratory Disease (FURD) is a complex disease characterized by, but not limited to, conjunctivitis, rhinitis, and tracheitis. Several pathogens encompass this disease including Feline Rhinotracheitis Virus (FHV), Feline Calicivirus (FCV), *Chlamydophila felis* (*C. felis*), *Bordatella bronchiseptica* (*B. bronchiseptica*), and *Mycoplasma felis* (*M. felis*). This disease process significantly effects cats in group housing situations such as animal shelters and is a leading cause of morbidity and mortality in these cats. Previous research has investigated the epidemiology of pathogens and risk factors in several geographical areas, the development of molecular diagnostic tools for the detection of pathogens, and the detection of previously unreported pathogens in this complex. This thesis encompasses three papers meant to address the objectives of atypical Mycoplasma detection in cats with and without FURD, validation of a field deployable molecular device for pathogen detection, and an overall epidemiologic evaluation of the unique Midwestern geographical area.

The first paper investigated the hypothesis that *Mycoplasma* species occur in the cat at higher levels of diversity than previously appreciated utilizing the unique molecular based Pan-Myco SYBR qPCR assay for the detection of the *Mycoplasma* genus and may be contributing to FURD. Overall, 15 *Mycoplasma* species were detected including: *M. gateae/canadense/arginini* cluster, *M. canis*, *M. hyorhinis*, *M. alkalescens*, *M. cynos*, *M. faucium*, *M. dispar*, *M. buccale*, *M. spumans*, *M. hominis*, *M. bovis*, *M. bovoculi*, *M. maculosum/leopharyngis* cluster, 2 new unknown species, and *Acholeplasma laidlawii*.

Twelve of these *Mycoplasma* species were previously unreported in cats and several of these species are potential zoonotic pathogens.

The second paper implemented a 5 stage pipeline for development and validation of field deployable assays utilizing the Insulated Isothermal Polymerase Chain Reaction (iiPCR) on the candidate field deployable device, POCKIT™ for the detection of FURD including FHV, FCV, *C. felis*, *M. felis*, and *B. bronchiseptica*. Thirty of both positive and negative clinical samples and surrogate samples were randomized, blinded, and tested side-by-side on both platforms for each pathogen. Limits of detection were biologically relevant, were equal to or less than 10 infectious units, and demonstrated near equivalency with clinical samples for all pathogen targets. Exclusivity testing demonstrated the iiPCR to be pathogen and target specific. Sensitivity and specificity for clinical samples ranged from 80%-97% and 93-100% respectively. Kappa values ranging from 0.80- 0.93 demonstrated strong agreement. Results demonstrate exceptional performance of the iiPCR reagents for detection of feline respiratory pathogens in clinical samples. This study demonstrates the effectiveness of the iiPCR for detection of feline pathogens and the use of the stages 1 through 3 of the pipeline for validation of field deployable reagents.

The final paper assessed detection of FURD pathogens in ocular, nasal, and oro-pharyngeal samples from cats in a specific unique geographical region and evaluated their association with disease in a cross-sectional study. There were 71 cats with respiratory disease and 119 cats without disease were enrolled in this study. Prevalence of FHV, FCV, *C. felis*, *Bordetella* species, *M. felis*, *M. gateae* cluster, and other *Mycoplasma* species in the total study population was 49%, 19.5%, 3.2%, 23.7%, 40%,

24.7%, and 15.3% respectively. Multivariate modeling showed that detection of *Bordetella* species (odds ratio (OR) = 3.143), FCV (OR = 2.830), and housing cats in shelters for 2 to 6 months (OR = 0.146) were all significantly related to FURD severity. Age of cats and detection of *C. felis* were confounding factors for relationship between *Bordetella* species and respiratory disease.

Taken in total, this research illustrates the necessity for continued research in the field of FURD. The discovery of previously unreported pathogens in felines, the application of new and emerging detection platforms for triage of disease outbreak, and the continued exploration of pathogen association and risk factors all contribute to the management of this disease in shelters. The impacts and translation of this research will help to prevent the high morbidity and mortality in shelter cat populations.

CHAPTER 1: GENERAL INTRODUCTION

This thesis focuses on the microbiological epidemiology of the feline respiratory tract in both healthy and diseased states in Midwestern shelter cats. Studies of Feline Upper Respiratory Disease in cats are performed to better understand the disease complex, predisposing factors, pathogens involved, and methods to detect those pathogens in an effort to better control respiratory disease in shelter populations.

Background

According to a survey of American pet owners in 2013, 68% of U.S. households owned a pet and 45.3 million of those households owned cats[1]. The total number of cats owned in the US was reported to be 95.6 million[1]. In 2012 it was reported that there were 6 to 8 million cats and dogs entering shelters across the U.S. each year with only 3 to 4 million being adopted from those shelters[2]. Additionally while 30% of lost dogs are reclaimed from shelters by their owners, only 2-5% of lost cats are reclaimed[2]. These numbers suggest that in the U.S. cats entering shelters have a high rate of mortality.

In 2006, a large needs-based assessment was performed in the western United States. The survey involved animal shelters in six states including responses from 78 shelters, which represented over 150,000 animals. This study reported that one of the three diseases of most concern for shelters and their feline populations was Feline Upper Respiratory Disease (FURD)[3]. FURD is a disease complex, similar to other respiratory disease complexes in domestic animal species, caused and compounded by the presence of several pathogens with many different predisposing and risk factors for disease.

While many of the risk factors for disease such as stress, high population density, and mixed age groups are rarely issues faced by household owned cats, cats entering animal shelters face all of these risk factors. These risk factors and others in combination with exposure to pathogens make animal shelters prime locations for respiratory disease outbreaks. All too often respiratory disease is endemic within a shelters feline population. For this reason, FURD is generally considered to be the leading cause of feline death in shelters and a leading cause of death in young cats overall[4].

Goals and Objectives

The overall goal of this collection of research was to improve the health and wellbeing of cats in shelter environments through increases in the general knowledge of FURD. This included education regarding the risk factors, associated pathogens, and translational application of pathogen detection assays for feline infectious agents. This goal was achieved through three specific objectives:

1) Translation and application of a broad pathogen detection assay to assess for the presence of previously unreported *Mycoplasma* pathogens in the feline upper respiratory tract and their association with respiratory disease,
2) To implementation of a 5 stage validation pipeline for the development of field deployable diagnostics using FURD pathogens proof of concept with an point of need assays and a platform which could be implemented in a shelter environment, and
3) Determination of the specific risk factors and pathogens contributing to FURD in Midwest shelters.

Thesis Organization

This research based thesis will encompass three complete manuscripts and follow the journal paper format. Chapter 1 provides a brief introduction to the feline overpopulation problem in the United States and subsequently the importance of FURD research for domestic cats in shelter environments. This introduction provides both the rationale for the following studies and also the goals and objectives of the overarching research plan. Chapter 2 provides a literature review which includes a brief overview of the main pathogens associated with FURD, the evidence regarding risk factors associated with FURD, and finally a review of the current body of literature on the prevalence of FURD and associated pathogens in shelter cats.

Chapter 3 is a manuscript titled 'Identification of Diverse and Unique Mycoplasma Species in the Feline Upper Respiratory Tract Utilizing the Pan-Myco SYBR Real-Time PCR Assay' for submission to *Plos Pathogens*. Chapter 4 is a manuscript titled 'Validation of a portable molecular diagnostic device for detection of pathogens associated with feline upper respiratory disease in cats' for submission to *The Journal of Veterinary Diagnostic Investigations*. Chapter 5 is a manuscript titled 'Feline Upper Respiratory Disease Pathogens in Midwestern Shelter Animals: a cross-sectional study' for submission to *Preventive Veterinary Medicine*. Chapter 6 is then comprised of general conclusions from this body of research. As this paper follows the journal paper format, references included in a chapter will be located at the end of that chapter and will either follow the formatting guidelines for the intended journal of submission or that of *Plos* for those chapters with general content.

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CHAPTER 2: LITERATURE REVIEW

Feline Upper Respiratory Disease Pathogens

Feline Upper Respiratory Disease is a complex which has many contributing pathogens. These pathogens serve as both primary and secondary invaders and work in conjunction to produce the severity and diversity of clinical signs.

Feline Herpesvirus-1

Feline Herpesvirus-1 (FHV) accounts for around half of the viral feline upper respiratory infections which are diagnosed [1]. FHV is a member of the *Alphaherpesviridae* subfamily which encompasses a majority of the human and animal herpesviruses. FHV is an enveloped double stranded DNA virus which only replicates in feline cells, including both domestic and wild cats [1,2]. One characteristic of alphaherpesviruses, shared by FHV, is viral persistence in a latent state in nervous and lymphoid tissues [1]. The virus is considered to be endemic in the domestic cat population with over 90% of cats being found serologically positive and the majority remaining latent throughout life [3]. FHV is not a hardy virus and does not survive for long periods in the environment. It is also susceptible to a large number of disinfectants including quaternary ammonia compounds [4]. FHV is spread through direct contact, fomites, and aerosolized droplets created through sneezing which can travel up to 1.3 meters [5].

Maternal immunity to FHV is lost around 2 months of age. After that time, FHV can induce acute severe upper respiratory tract disease characterized as feline viral rhinotracheitis. It has been experimentally shown that installation of 10^2 to 10^7 $CCID_{50}$ of

FHV in the nostrils will reliably reproduce disease [6]. After oro-nasal inoculation with FHV, the virus incubates for 2-6 days [1], replicating in the upper respiratory mucosa causing clinical signs. Infected cats will begin to shed virus after 24 hours and continue for 1 to 3 weeks [7]. Clinical signs include pyrexia, anorexia, sneezing, and serous nasal discharge for 10 to 14 days [4]. FHV has cytolytic properties which can result in oral mucosal ulceration as well as dendritic ulcers in the cornea, considered pathognomonic for this virus [1]. Conjunctival hyperemia, serous ocular discharge, and chemosis can also be present [1,7]. In cats, a reoccurrence of active infection can be induced by stress, including rehoming, or administration of corticosteroids [8,9].

Treatment for FHV infection in cats is mainly focused on supportive care and prevention of secondary bacterial infections. Antiviral therapy is most effective in cats when supplied topically using Idoxuridine, a drug which is well tolerated by cats and has high clinical efficiency [5]. Oral supplementation with L-Lysine has also been shown to reduce the severity of disease both during initial infection and after recurrence from latency [10,11]. FHV is considered to be a component of core vaccination schedules for cats and is included in several combination vaccine products called “FVRCP”s [1]. The American Association of Feline Practitioners recommends the use of modified live vaccination on a schedule of a single dose at intake to a shelter environment and depending on the age of the cat, either one booster 2 to 3 later or every 2 to 3 weeks until 16 weeks of age [12]. Other preventative measures include isolation of all cats at intake, isolation of all cats with clinical symptoms, and prevention of aerosol transmission through barriers between cats [7].

Feline Calicivirus

Feline Calicivirus (FCV) is a highly variable single-stranded RNA virus in the family *Caliciviridae* and the genus *Vesivirus*. FCV infects both domestic and wild cats and has not been reported to be zoonotic or pathogenic to other domestic animal species [2,13]. The capsid protein is the most variable region of the virus, the target for the immune system, and allows for wide viral variability [17]. FCV has been proven to cause respiratory disease in cats through experimental infection [14-16]. FCV causes a variety of clinical signs and syndromes with the variability in strains leading to a large spectrum of disease severity and clinical manifestations [17]. It is the second important viral cause of FURD as well as chronic stomatitis, lingual ulceration, and transient limping [17-19].

FCV is widespread in feline populations of cats with and without clinical symptoms. Maternal antibody persists for up to 14 weeks but kittens become susceptible to FCV infection earlier [20]. FCV is transmitted through the respiratory mucosa (oral, nasal, and conjunctival) and replicates in the oropharynx [21]. Experimental infection demonstrated that an infectious dose of 10^4 $TCID_{50}$ caused serous nasal and ocular discharge, minimal sneezing, and lingual ulcers [16] Once in the host, FCV becomes viremic, often with associated pyrexia, and the virus causes necrosis of epithelial cells resulting in clinical signs such as oral ulceration [21]. Depending on the strain oral and respiratory signs may give way to limping and acute arthritis [22,23]. FCV also has a more virulent systemic variant which causes vasculitis, edema, and jaundice. In severe cases FCV can cause hemorrhage with mortality rates as high as 40% [24-26]. This variant is more severe in adult cats. After a cat recovers from acute infection, the virus

can be shed by that cat for up to 30 days or longer and may represent a persistent carrier state [21,27].

Due to the severity of disease that FCV can cause, infected cats with clinical signs may require intensive nursing and supportive care. With cats, especially with diseases that cause oral pain, it is very important to provide nutrition, via palatable feed or placement of feeding tubes, to prevent the development of hepatic lipidosis [21]. Non-steroidal anti-inflammatories can also be used to address the pyrexia and oral pain involved in infection [21]. Because FCV is a RNA virus, current antiviral therapies have limited efficacy. Feline interferon-omega, used for treatment of Feline Leukemia Virus, has been demonstrated to be efficacious against FCV. Feline interferon-omega inhibits FCV viral replication in-vitro and improved both pain and oral lesions in cats [28,29]. FCV is considered to be a core vaccine component for cats [12]. FCV is commonly a component of the "FVRCP" vaccine and the recommended vaccination protocol is the same as previously discussed for FHV. If disease occurs in a fully vaccinated cat a different vaccine should be used as infection may have occurred with a different strain of the virus [12].

FCV can persist in the environment for up to a month and is shed for long periods of time [21]. Early disease detection, isolation, and proper disinfection are essential in shelter environments to limit the spread of disease. FCV has been shown to be susceptible to sodium hypochlorite, chlorine dioxide, and potassium peroxymonosulfate disinfectants, but not to quaternary compounds [4]. Thus it is important to make sure that a shelter is utilizing chemicals that will remove the virus from the environment effectively.

Chlamydophila felis

Chlamydophila felis (*C. felis*) is an obligate intracellular gram negative bacteria which has a unique infectious cycle involving reticulate and elementary bodies which are released upon cell lysis, with the elementary bodies being infectious [30]. *Chlamydia* species share close homology and *C. felis* has been reported to have zoonotic transmission to humans from cats [31-34]. *C. felis* antibodies have also been reported in civets and bearcats [35]. *C. felis* has been proven to cause upper respiratory disease and conjunctivitis in cats through experimental infection [36-38]. One study determined that an infectious dose of $10^{2.8}$ $CEID_{50}$ reproducibly causes both unilateral and bilateral conjunctivitis [38].

Most *C. felis* infections occur in cats less than 1 year of age and *C. felis* is spread through direct or close contact between cats and ocular fluids [30]. Maternal antibody protects kittens from 1 to 2 months of age [30]. Once on host mucosal tissues, *C. felis* requires 2-5 days for incubation after which point acute unilateral conjunctival chemosis and hyperemia can be seen [30]. Unilateral disease normally progresses to bilateral disease and serous to mucoid ocular discharge, blepharospasm, and discomfort are characteristic [30]. Transient pyrexia, anorexia, and lethargy can also accompany infection. Conjuctival shedding of the organism can exceeded 60 days [38].

Several studies have shown that *C. felis* is effectively treated through systemic use of Doxycycline at a 10 mg/kg/day dose [39-41]. Potentiated Amoxicillin is safe treatment with few side effects and may be best for young kittens [30] but Doxycycline therapy for at least 28 days is preferred [41]. Cats treated with potentiated Amoxicillin may have

recurrence of clinical signs and require additional therapy [40]. *C. felis* is not considered a core-vaccine in cats but instead a vaccine given once pathogens have been isolated in the population and risk assessment suggests it will be helpful for disease control [12]. One study utilizing a live *C. felis* vaccine demonstrated that vaccination decreased clinical signs but did not affect the shedding of infectious particles [42]. Vaccination schedules for *C. felis* begin between 8 to 12 weeks of age and require a booster 3 to 4 weeks later with a yearly booster for cats with exposure [30]. *C. felis* detection in cats in shelter environments is not as common as the viral causes of FURD [43]. This bacteria is not thrifty in the environment and routine hygiene utilizing quaternary ammonia dilutions is sufficient [32].

Bordetella bronchiseptica

Bordetella bronchiseptica (*B. bronchiseptica*) is a gram negative coccobacillus with a wide animal host range including cats, dogs, swine, and rabbits [44]. Additionally, *B. bronchiseptica* has been implicated in several zoonotic transmissions from cats to humans and specifically a concern for immune-compromised pet owners [45-47]. Transmission has also been reported between domestic animal species and passage has been documentation from a dog to a cat [48]. These transmissions are especially important when considering shelter housing where cats and dogs may be exposed to one another and shelter personnel are exposed to both species [49]. Experimental infection of cats via aerosol methods and intranasal injection with $10^{7.5}$ colony forming units (CFU) has reliably produced disease [50-52].

B. bronchiseptica is shed in the nasal and oral secretions of cats and spread through direct and indirect contact [53]. Maternal antibody against *B. bronchiseptica* is low in kittens and may only last for 2 to 8 weeks post-partum [50,52]. Clinical signs cause by infection range from mild pyrexia, coughing, sneezing, and ocular discharge to more severe disease such as pneumonia, cyanosis, and dyspnea [44,50,52,53]. Disease may be more severe in kittens under 10 weeks of age [44]. One of the distinguishing features of *B. bronchiseptica* infection is it is one of only a few infectious causes of feline cough. Clinical signs average 10 days with bacterial isolation possible up to 10 weeks post infection, suggesting that animals may be shedding bacteria long after the resolution of clinical signs[44].

Antimicrobial therapy is important in the case of *B. bronchiseptica* infection to prevent the extension of upper respiratory disease into the lower respiratory tract resulting in pneumonia [44]. When possible, bacterial culture and sensitivity should be performed to assess for appropriate antimicrobial as several studies have shown that *Bordetella* species have varying resistance. Tetracycline drugs appear to have the lowest percentage of resistance (1.3-2%), while resistance to potentiated Amoxicillin was much higher (14.5%) [54,55]. A study of disinfectants utilized for swine facilities suggests that *B. bronchiseptica* is highly susceptible to most common disinfectants as long as organic material is removed first [56]. *Bordetella* vaccination is not considered a core-vaccine for cats and vaccine recommendations for shelters are the same as those for *C. felis* [12].

Mycoplasma felis

Mycoplasma felis (*M. felis*) is a prokaryotic organism from the class Mollicutes which lacks a cell wall and survives on mucosal surfaces where it acts as both primary and commensal opportunistic pathogen [57]. *M. felis* infects both domestic and wild cats as well as horses [58,59]. Zoonotic transmission from cats to humans has also been reported through a cat bite and increased feline exposure [60,61]. Experimental infection of kittens with 10^7 CFU in both the nostrils and conjunctiva reliably produced disease [62,63].

Infection with *Mycoplasma* can occur through respiratory aerosols and reproductive secretions from infected animals. In group housing *Mycoplasma* can also be transmitted through fomites and close contact [64]. Once infected, *M. felis* can cause conjunctivitis, ocular and nasal discharge and anorexia [62,65]. *M. felis* can also cause chronic rhinosinusitis [66], ulcerative keratitis [67], and can progress into pneumonia and arthritis [68,69]. Treatment of *Mycoplasma* infection is effectively performed using Pradofloxacin or Doxycycline[70]. One study showed that an extended 14 day treatment regimen of Doxycycline was most effective for reduction of DNA detection [71].

Several species of *Mycoplasma* have been reported as being able to form biofilms and have also been shown to survive for extended periods when dried onto surfaces [72]. Thus they can become environmental contaminants and persist on fomites. Quaternary ammonium compounds have not been shown to be effective against *Mycoplasma* species but 70% Ethanol and alkaline cleaners as well as bleach products have been effective for killing *Mycoplasma* with a 5 minute contact time[73]. There is currently no vaccine

available for *M. felis* and measures such as regular disinfection, isolation of sick animals, and reduction of respiratory aerosols are the mainstays of infection prevention.

Evidence for Predisposing Factors

Many studies of FURD pathogens also included data on predisposing risk factors and protective factors that influence the development, progression, and maintenance of disease.

Age

Several studies have reported that younger animals are at greater risk for developing FURD [43,74-76]. This may be due to the waning of maternal antibody, the stresses of weaning, and the lack of vaccination histories of both the queen and the kittens. The risk is also significantly increased for cats greater than 11 years of age [75]. Older animals may have additional health concerns which increase their stress levels as well as impair their immune systems. Additionally, animals surrendered to shelters at an older age may have greater stress induced by rehoming. The results in these studies are influenced by the ages that sampling included and the ability to determine age of cats based on records or dental analysis. Determining the age of cats with dental records after two years of age is fairly subjective. This is because all teeth have erupted at this point and the scale utilized by most shelters requires evaluation of dental plaque and color [77,78].

Sex and sterilization status

While several studies agree that there is no sex predisposition to FURD [43,79,80], Studies disagree on the influence of spay/neuter surgery. One study found intact cats to have a higher incidence of disease[74] , another finding neutered males at higher incidence [81], and another finding the incidence of FURD to be lower in spayed female cats as compared to male cats [75]. These findings can be complicated by the fact that younger animals coming into shelters tend to be intact and older animals coming into shelter populations are more likely castrated [81]. Additionally, the reporting of reproductive status cannot always be determined in female cats unless an obvious spay incision is visible or palpable. With the recent trend of pediatric spaying, identification of spayed females is more difficult and implementation of tattooing post-surgery is essential.

Source

Sources evaluated previously generally include either stray animals or owner surrender animals. Stray cats are generally found to have a higher incidence and risk of developing FURD than owner surrender cats in the same populations [75,81,82]. One study reported a 17% increase in risk for stray cats [81]. Owner surrendered cats are more likely to have a vaccine history and have received preventative medicine and less likely to have concurrent immune-suppressive diseases such as Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) on presentation to shelters which may contribute to this difference in incidence of disease. Conversely, owner surrender cats are likely to be middle aged to older cats accustomed to one housing situation and more

susceptible to higher stress in the shelter environment. More research is needed to determine the different contributing factors for these two cat populations and their individual risks.

Breed

Although it may be difficult to determine a cats breed on entry to the shelter, one study found that purebred cats were at a higher risk of developing FURD than mix breed cats [75]. This result may have been skewed by the small number of purebred cats included in the study and the fact that 1/3 of the purebred cats were kittens. Another study found the highest detection of FHV and FCV in domestic shorthair cats [74] while a second study found purebred cats to be significantly at risk for development of FURD [76]. These conflicting results are likely due to the number and definition of purebred cats included in the study populations.

Vaccination history

While many studies record whether cats were vaccinated upon arrival to the shelter and whether cats had vaccine histories on intake, only one study remarked on whether this was significant. This study reported that FURD was more common in unvaccinated cats [74].

Time in shelter

Several studies agree that the longer the time spent in the shelter the higher the risk of FURD [43,75]. Studies have shown that being housed in the shelter longer than 6 days increased FURD risk [43] and that FURD incidence remains low until day 6 and

between day 6 and 13 it rises to almost 80% incidence [75]. Conversely, it has also been shown that the risk of FURD decreases over time spent in the shelter [76]. The study suggests that early exposure and disease occur within the first 6 to 12 days in the shelter followed by latency and remission of disease [76]. In this study, the majority of FURD infections occurred within the first 50 days in the shelter [76]. The study of 'time in shelter' is complicated by the fact that most shelters, unless they are no-kill, do not hold cats for extended periods of time. In addition, population turnover is high and dictated by adoption, foster, and euthanasia.

Hygiene

Two studies demonstrated that a decrease in hygiene of the feline environment was associated with an increase in FHV, FCV, *C. felis*, and *B. bronchiseptica* [49,83]. As several of these pathogens have been shown to be stable in the environment in respiratory exudates and on fomites, an environment without proper cleaning protocols would lead to a buildup of pathogens and an increase in the infectious dose to which animals are exposed.

Presence of dogs

Cats housed in close proximity to dogs have also been shown to be at increased risk of developing FURD [49,83]. As transmission between canines and felines of *B. bronchiseptica* has been demonstrated previously, the spread of these bacteria between closely housed cats and dogs may be the cause of this relationship. Additionally, cats rehomed in shelters that had previously never been housed with dogs may experience an increase in stress level when exposed to dogs and canine related noise.

Season

One study found that the peak incidence for reported disease caused by FHV and FCV was during the winter months[74]. This effect can be explained by the stability of both viruses at lower temperatures, the stress winter time may put on cats, and the fact that owners may spend more time indoors and notice milder clinical signs [74,84].

Review of the Prevalence of FURD and FURD Associated Pathogens in Shelter Cats

Several studies have been done around the world assessing both the prevalence of FURD and associated pathogens. The objective of this section of the literature review is to conduct a critical comparative review of the current literature of prevalence estimates for both FURD and the detection of associated pathogens in the shelter environment.

Review of prevalence studies in literature:

Literature review was conducted in two portions. First review was performed for Feline Upper Respiratory Disease (FURD). The case definition used for FURD was for reporting of prevalence estimates in shelter animals displaying clinical signs of upper respiratory disease. The target population was specifically cats housed in shelter environments and the definition of FURD addressed displays of clinical signs including sneezing, ocular discharge, nasal discharge, and oral lesions.

The second review was for the individual pathogens which can be etiologic agents of FURD including FHV, FCV, *C. felis*, *B. bronchiseptica*, and *Mycoplasma* species. For each of the pathogens, the target condition was the prevalence of detection of the pathogen in feline upper respiratory samples (i.e. ocular, nasal, or oro-pharyngeal

samples). Due to the many different diagnostic tests available per pathogen, isolation and/or detection was the target condition and substantial replication or histopathology was not necessary as several of these pathogens have had reported latent phases and the widest target definition was utilized.

A review of prevalence studies for both topics was conducted on February 26, 2014. Studies from 1990 to the present were included in the search parameters. Search occurred on the web based databases Web of Knowledge, PubMed, and Google Scholar. The search terms were: Feline/cat Upper Respiratory Disease combined with shelter. Additional searches were performed for individual pathogens using the search terms individual pathogen name, feline/cat, shelter, and respiratory. When searching for the pathogen *C. felis* the search term conjunctivitis was also included.

Data extraction and quality assessment

Data extraction was performed in several steps. First all articles which were reviews of disease and not specifically novel reporting from shelters were eliminated. Additionally any reports that were clinical trials of antimicrobials, vaccines, or other therapies were also excluded. Publications not reporting prevalence and publications reporting a single animal case study or lethal outbreak situation were excluded. Studies prior to 1990 were excluded and data from cat populations other than shelter animals were excluded.

The following data was collected from the remaining publications: country and region, year of study, FURD or specific pathogen prevalence reported (some articles reported both and were included in both analysis), test utilized for detection/isolation of

pathogens/determination of clinical status, study population size and age distribution, anatomical sampling location, and for pathogen prevalence what was the clinical FURD condition of the cats included. The majority of studies failed to report or vaguely reported much of the information sought and the overall quality of the studies was poor. One study was removed due to an inability to determine the prevalence reported for the agents [85] and two others were removed because the shelter populations studied could not be distinguished from other non-shelter populations of cats in the studies[83,86].

The majority of papers had critical issues regarding collection of relevant information. Highest among these was the lack of reporting of test accuracy, or sensitivity and specificity. Because different papers utilize different testing platforms with different accuracy levels, knowing the sensitivity and specificity of the testing performed allows for the calculation of true prevalence (TP) from the reported prevalence (RP). TPs then allow for the comparison of prevalence findings across reports and assay types. Additionally, for many of the studies, the selection of cats included in the study populations was not random which resulted in selection bias. For the included studies, reported critical issues were also noted and placed into one of several categories which were: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred; (e) no definition was provided for what constitutes a positive detection of target pathogen; (f) no sensitivity or specificity information provided for assay. These critical issues are reported along with other data collected as

indicated by the letter assigned the category. Critical issues with the reported studies were taken into consideration when determining whether a prevalence estimate from a study could be compared to other studies.

Data Analysis

The AP reported was either determined through reporting of the individual study or was calculated as the number of animals positive for a target out of the total number of animals tested for the target. The definition of test positive was determined by the individual studies and frequently what constituted a positive was not reported (i.e. what was the PCR cut-off for positive values, etc). Tests used to determine FURD status in studies were all subjective and based on clinical exam findings [75,76,79,81] and while every study utilized similar clinical symptoms, no validation study was performed on the scoring systems. TPs were calculated using the Rogan-Giaden estimating formula as reported in a previous study[87]. Microsoft Excel ™ was utilized to perform the formula:

$$TP = (AP + Sp - 1) / (Sp + Se - 1).$$

For assays which had no sensitivity or specificity reported, sensitivity and specificity sensitivity and specificity for the same platform and a highly related target were utilized. For example, as no sensitivity and specificities were able to be determined for FHV for viral isolation, viral neutralization, and PCR, assay sensitivity and specificities as determined for Herpes Simplex Virus (HSV), another *alphaherpesvirus*, were utilized instead [88]. The sensitivity and specificity for testing for *C. felis* was performed using those determined for *Chlamydophila* species in cattle [89]. *B. pertussis* assay measures were substituted for *B. bronchiseptica* measures as they are closely

related *Bordetella* species[90]. All included *M. felis* reports utilized polymerase chain reaction (PCR) platforms and as such no TP was calculated. No sensitivity and specificity data could be found for FCV assays and as the FCV virus is highly variable no surrogate target was used and no TPs were calculated.

Results

Literature search

Initial searches performed resulted in 5449 hits (5390 on Google scholar, 37 on Web of Knowledge, and 22 on PubMed). After exclusion of repeat records, records that did not include shelter populations, records which were vaccine or antibiotic trials, and records which were conference proceedings 16 publications remained. Of these 2 were excluded because the shelter population could not be separated from the general study population and 1 was excluded because prevalence could not be determined from the reported results.

The remaining 13 publications were included in this prevalence study. The publications compromise studies from across the United States, Canada, the United Kingdom, Germany, Belgium, and Korea. Of these studies, 4 reported on overall FURD prevalence in a shelter and 1 of these papers also reported individual target prevalence [81]. There were 8 studies which reported FHV prevalence, 8 reporting FCV prevalence, 5 reporting *C. felis* prevalence, 6 reporting *B. bronchiseptica* prevalence, and 4 reporting *Mycoplasma* species prevalence.

FURD in shelters

A summary of the 4 shelters reporting overall FURD prevalence in shelters is presented in Table1.1. This table includes information on the location and time period of the study, the clinical definition of FURD used by the study, the reported prevalence of FURD, the study size, the distribution of feline age in the study, and the reference. As the reported prevalence in each of these studies was based on subjective clinical scoring of disease in the cats, no TP was calculated.

Table 1.1. Summary of the prevalence of Feline Upper Respiratory Disease in shelter cat populations in studies published from January 1990 to February 2014.

Country/ Region	Study period	Clinical definition	RP (%)	n	Age distribution	CI[1]	Reference
Canada/ Vancouver	2010	Undefined	33.60	250	6 mo -8 yr	a,b	Gourkow et al, 2013
USA/ Northeast	2009- 2010	Listed signs	30.00	2734	kittens and older	c	Dinnage et al, 2009
UK	2002- 2003	Scoring system	4.10	1434	3-72 mo	c,d	Edwards et al, 2008
UK	1994 - 1995	Scoring system	36.80	185	1 mo – 7+ yr	c,d	Binns et al, 2000

Abbreviations: RP, Reported Prevalence; n, number of cats tested; CI, Critical Issues.
[1]Critical issues: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred.

Pathogens in shelters

Table 1.2. presents a summary of the 8 prevalence studies on FHV in shelter environments. Table 1.3. presents a summary of the 8 prevalence studies of FCV. Table 1.4. presents a summary of the 5 prevalence studies of *C. felis*. Table 1.5. presents a

summary of the 6 prevalence studies of *B. bronchiseptica*. Table 1.6. presents a summary of the 4 prevalence studies of *Mycoplasma* species. Each table includes a summary of the location and time period of the study, the anatomical sampling location, the testing platform and detection target, the reported prevalence, sensitivity and specificity of the testing platform (with the exception of FCV and *Mycoplasma* species), the calculated true prevalence (when performed), the total study size, age of distribution, what percentage of the pathogen positive animals had FURD, and any critical issues present in the study.

Critical review of the prevalence of FURD

All of the studies which reported a prevalence of FURD were given critical comments, as reported in Table 1.1. One study did not provide a definition for the outcome FURD and only reported that it was determined [81]. This study also did not clearly state the criteria for inclusion of a cat in the study and it was therefore impossible to determine if selection bias had been introduced in the study [81]. Three studies were designed in such a way that multiple observers recorded disease scores for the cats in the studies. None of these studies remarked on inter-observer agreement and the possible reporting bias created by using multiple observers [75,76,79]. One paper did comment on specific training that all observers received [76]. One study reported that, while the design of the study involved sampling all animals taken into a shelter, this practice was not implemented and selection

Table 1.2. Summary of the prevalence of Feline Herpes Virus in shelter cat populations in studies published from January 1990 to February 2014.

Country/ Region	Sampling site	Test	Target	RP (%)	Se	Sp	TP	n	Age distribution	% with FURD	CI[1]	Reference
Canada/ Vancouver	Conjunctival/ oral-nasal	PCR	Glycoprotein B	2	1	0.78	2.28	250	6 mo -8 yr	100	a,e,f	Gourkow et al, 2013
USA/ Florida	Blood	VN	Antibody	11	0.81	0.99	13.79	347	>6mo – 5+yr	NR	f	DiGangi et al, 2012
USA/ California	Conjunctival/ Deep laryngeal	PCR	Glycoprotein B	78.2	1	0.78	99.7	101	NR	89.9	a	Burns et al, 2011
Germany	Conjunctival	PCR	Not reported	25	1	0.78	31.68	20	3 mo - 16 yr	100	d,e,f	Hartmann et al, 2010
Belgium/ Liege	Oral	PCR	Glycoprotein gC	30.1	1	0.78	38.2	299	2-180 mo	NR	e,f	Zicola et al, 2009
Korea/ Yangju	Conjunctival/ oro-pharynx	PCR	TK gene	63	1	0.78	80.26	78	NR	0	d,e,f	Kang, et al 2008
USA/ California	Conjunctival/ oropharyngeal	VI	CPE	23.4	0.81	1	28.92	573	0-96+ mo	70	e,f	Bannasch et al, 2004
USA/ California	Oropharyngeal	VI	CPE	0	0.81	1	0	42	NR	NR	f	Foley et al, 2002

Abbreviations: PCR, Polymerase Chain Reaction; VN, Viral Neutralization; VI, Virus Isolation; CPE, Cytopathic Evidence; RP, Reported Prevalence; Se, Sensitivity; Sp, Specificity; TP, True Prevalence; n, number of cats tested; CI, Critical Issues; NR, Not Reported.

[1]Critical issues: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred; (e) no definition provided for what constitutes a positive detection of target pathogen; (f) no sensitivity or specificity information provided for assay.

Table 1.3. Summary of the prevalence of Feline Calicivirus in shelter cat populations in studies published from January 1990 to February 2014.

Country/ Region	Sampling site	Test	Target	RP (%)	n	Age distribution	% with FURD	CI[1]	Reference
Canada/ Vancouver	Conjunctival/ oral-nasal	PCR	ORF 1	2.8	250	6 mo -8yr	43	a,e,f	Gourkow et al, 2013
USA/ Florida	Blood	VN	Antibody	36.6	347	>6mo - >5 Yr	NR	f	DiGangi et al, 2012
USA/ California	Conjunctival/ deep laryngeal	PCR	ORF 1	12.9	101	NR	76.9	a	Burns et al, 2011
Belgium/ Liege	Oral	RT-PCR	p30 + VP1	43.1	299	2-180 mo	NR	e,f	Zicola et al, 2009
Korea/ Yangju	Conjunctival/ oro-pharynx	PCR	Capsid protein	0	78	NR	0	d,e,f	Kang et al, 2008
UK	Oropharyngeal	VI	CPE	28	116	NR	NR	c,f	Coyne et al, 2007
USA/ California	Conjunctival/ oropharyngeal	VI	CPE	28.1	573	0-96+mo	56	e,f	Bannasch et al, 2004
USA/ California	Oropharyngeal	VI	CPE	52.4	42	NR	NR	f	Foley et al, 2002

Abbreviations: PCR, Polymerase Chain Reaction; VN, Viral Neutralization; VI, Virus Isolation; CPE, Cytopathic Evidence; RP, Reported Prevalence; Se, Sensitivity; Sp, Specificity; TP, True Prevalence; n, number of cats tested; CI, Critical Issues; NR, Not Reported.

[1]Critical issues: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred; (e) no definition provided for what constitutes a positive detection of target pathogen; (f) no sensitivity or specificity information provided for assay.

Table 1.4. Summary of the prevalence of *Chlamydophila felis* in shelter cat populations in studies published from January 1990 to February 2014.

Country/ Region	Sampling site	Test	Target	RP(%)	Se	Sp	TP[1]	n	Age distribution	% with FURD	CI[2]	Reference
Canada/ Vancouver	Conjunctival/ oral-nasal	PCR	OmpA	0.4	1	0.99	0.39	250	6 mo -8 yr	100	a,e,f	Gourkow et al, 2013
USA/ California	Conjunctival/ deep laryngeal	PCR	OmpA	0.01	1	0.99	0	101	NR	0	a	Burns et al, 2011
Germany	Conjunctival	PCR	NR	35	1	0.99	35.34	20	3 mo - 16 yr	NR	d,e,f	Hartmann et al, 2010
Korea/ Yangju	Conjunctival/ oro-pharynx	PCR	Outer membrane	0	1	0.99	-0.01	78	NR	0	d,e,f	Kang et al, 2008
USA/ California	Conjunctival/ oropharyngeal	PCR	NR	2.8	1	0.99	2.82	573	0-96+ mo	82	e,f	Bannasch et al, 2004

Abbreviations: PCR, Polymerase Chain Reaction; VN, Viral Neutralization; VI, Virus Isolation; CPE, Cytopathic Evidence; RP, Reported Prevalence; Se, Sensitivity; Sp, Specificity; TP, True Prevalence; n, number of cats tested; CI, Critical Issues; NR, Not Reported.

[1]TP falls between 0 and 100% and as such any values calculated as <0 or >100% should be considered to be 0 or 100% respectively.

[2]Critical issues: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred; (e) no definition provided for what constitutes a positive detection of target pathogen; (f) no sensitivity or specificity information provided for assay.

Table 1.5. Summary of the prevalence of *Bordetella bronchiseptica* in shelter cat populations in studies published from January 1990 to February 2014.

Country/ Region	Sampling site	Test	Target	RP (%)	Se	Sp	TP[1]	N	Age distribution	% with FURD	CI[2]	Reference
Canada/ Vancouver	Conjunctival/ oral-nasal	PCR	FhaB	2.4	0.935	0.971	2.62	250	6 mo -8 yr	83	a,e,f	Gourkow et al, 2013
USA/ California	Conjunctival/ deep laryngeal	PCR	FhaB	9.9	0.935	0.971	10.9	101	NR	80	a	Burns et al, 2011
USA/ California	Conjunctival/ oropharyngeal	Culture	Whole target	9.5	0.152	1	62.5	573	0-96+ mo	57	e,f	Bannasch et al, 2004
USA/ California	Oropharyngeal	Culture	Whole target	22.22	0.152	1	146.18	45	NR	NR	f	Foley et al, 2002
Belgium/ Flanders	Nasal	Culture	Whole target	4.6	0.152	1	30.26	65	<6mo to >6mo	NR	b,f	Pasmans et al, 2001
UK	Oropharyngeal +/- nasal	Culture	Whole target	19.5	0.152	1	128.29	185	1 mo – 7+yr	NR	d,f	Binns et al, 1999

Abbreviations: PCR, Polymerase Chain Reaction; VN, Viral Neutralization; VI, Virus Isolation; CPE, Cytopathic Evidence; RP, Reported Prevalence; Se, Sensitivity; Sp, Specificity; TP, True Prevalence; n, number of cats tested; CI, Critical Issues; NR, Not Reported.

[1]TP falls between 0 and 100% and as such any values calculated as <0 or >100% should be considered to be 0 or 100% respectively.

[2]Critical issues: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred; (e) no definition provided for what constitutes a positive detection of target pathogen; (f) no sensitivity or specificity information provided for assay.

Table 1.6. Summary of the prevalence of *Mycoplasma* species in shelter cat populations in studies published from January 1990 to February 2014.

Country/ Region	Sampling site	Test	Target	RP(%)	n	Age distribution	% with FURD	CI[1]	Reference
Canada/ Vancouver	Conjunctival/ oral-nasal	PCR	ITS-1	21.6	250	6 mo -8 yr	39	a,e,f	Gourkow et al, 2013
USA/ California	Conjunctival/ deep laryngeal	PCR	ITS-1	25.7	101	NR	76.9	a	Burns et al, 2011
Germany	Conjunctival	PCR	NR	25	20	3 mo - 16 yr	NR	d,e,f	Hartmann et al, 2010
USA/ California	Conjunctival/ oropharyngeal	PCR	16S rRNA	14.4	573	0-96+ mo	94	e,f	Bannasch et al, 2004

Abbreviations: PCR, Polymerase Chain Reaction; VN, Viral Neutralization; VI, Virus Isolation; CPE, Cytopathic Evidence; RP, Reported Prevalence; Se, Sensitivity; Sp, Specificity; TP, True Prevalence; n, number of cats tested; CI, Critical Issues; NR, Not Reported.

[1]Critical issues: (a) no study definition of FURD was reported; (b) study had unclear explanation of inclusion criteria for cats and therefore selection bias could not be determined; (c) disease status was determined by more than one observer with no reporting of measures of inter-observer agreement; (d) study was either designed for non-random sampling or reported that non-random sampling occurred; (e) no definition provided for what constitutes a positive detection of target pathogen; (f) no sensitivity or specificity information provided for assay.

bias occurred, while a second study utilized convenience sampling which also incurs selection bias [76,79].

Three of the four studies have very similar reported prevalence and together the average reported prevalence is 33.47%. One study reports a prevalence of 4.1% FURD in the shelter population [76]. This is much lower than the other reported prevalence and may be explained by the fact that several shelters participating in the study did not report all of their cats and so selection bias was introduced. This is compounded by the fact that all personnel in the shelters were responsible for tracking and recording the feline information and so there were multiple people responsible for interpreting disease presence or absence.

Critical review of the prevalence of target pathogens

All of the pathogen target based prevalence studies received at least one critical comment. The majority of this is due to the fact that sensitivity and specificity of assays utilized in the studies were not reported and very few studies reported any validation steps taken to ensure assay performance. As described in above, due to this lack of reporting substitute sensitivities and specificities were utilized when appropriate for the determination of true prevalence.

Overall 8 studies qualified for inclusion in FHV prevalence analysis, 8 studies for FCV analysis, 5 studies for *C. felis* analysis, 6 studies for *B. bronchiseptica* analysis, and 4 studies for *M. felis* analysis. Three of these studies were included in all pathogen analysis [43,81,91]. The first of these studies was also utilized in the FURD prevalence review and did not provide a definition of FURD used in the study and utilized outside testing of samples through IDEXX and thus did not supply a sensitivity, specificity, or

definition for a positive vs negative test result[81]. The second of these studies did not supply a definition of FURD but did explain validation and performance requirements for the assays utilized as well as report Ct values for one of the PCR tests performed [91]. This paper also utilized outside testing through IDEXX reference laboratories but overall this paper had a much more thorough explanation of study design and methodology used [91]. The final overarching study had shelter personal select cats for inclusion in the study as cases and controls were then randomly selected which could lead to selection bias[43]. This study also utilized a mixture of bacterial culture, viral isolation, and PCR for identification of the different targets. While there is explanation for culture and viral results, there is no definition of PCR positive results or internal validation of the testing platforms[43].

Three studies included three of the target pathogens [92-94]. Hartmann et al. (2010) included the targets FHV, *C. felis*, and *Mycoplasma* species. The multiplex qPCR assay was previously utilized in a publication but no validation data was provided for the sensitivity and specificity of the assay for detection of FHV and *C. felis* [92]. They do perform a comparison between the Immunofluorescence Assay(IFA) and qPCR for *C. felis* but do this with the unknown clinical samples and thus the comparison does not serve as a validation of either method. The *Mycoplasma* species assay was sourced from a previous publication where validation of the assay was performed. Additionally there is no explanation of what constitutes a positive detection. Finally this study only included cats with clinical signs of conjunctivitis and selection bias occurred [92]. Foley et al. (2002) included the targets FHV, FCV, and *B. bronchiseptica*. This study was very complete in its methodology and all cats present at the time of sampling were included so

no selection bias occurred [93]. The study did not report sensitivity or specificity for the target isolation [93]. Kang et al. (2008) included the targets FHV, FCV, and *C. felis*. Cats were included in the study prior to euthanasia and were not a random sampling of the population[94]. In addition to selection bias, there was no definition of a positive target detection and sensitivity and specificity of the assays utilized were not reported [94].

Both DiGangi et al. (2012) and Zicola et al. (2009) reported prevalence for both FHV and FCV targets. Neither study reported sensitivity and specificity of the platforms used and Zicola et al. (2009) also failed to report what constituted a positive detection. While both studies randomly selected cats included in the study, selection bias was introduced in Zicola et al. (2009) as only cats in isolation were included in the study [80,95].

Three studies only reported on a single target. Coyne et al. (2007) reported on the prevalence of FCV in a single UK shelter, had several observers reporting FURD scores, and did not report the sensitivity and specificity of the viral isolation used for isolation [96]. Two of these studies only reported on *B. bronchiseptica* prevalence [49,97]. Neither of these studies reported sensitivity and specificity of their assays. Pasman et al. (2001) had unclear inclusion criteria for their cat population and Binns et al. (1999) utilized convenience sampling to recruit a non-random sampling of the cat population.

Discussion

Overall the prevalence of FURD reported in the studies included in this review ranged from 4.1-36.8% with 3 out of the 4 studies reporting very similar prevalence. The mean FURD prevalence was 26.13% (95% CI: 25.72, 26.53). This suggests that 1 out of every 3 cats coming into a shelter may either be entering with FURD already present or is

at risk of developing FURD. This also explains why FURD is such a burden on shelters as 1 out of every 3 cats in a shelter may require additional treatment, time in isolation, increased time in the shelter prior to adoption, and increased costs associated with all of those factors. Table 1.7. provides a summary for FURD and all of the targets and their RPs and calculated TPs along with 95% confidence intervals.

Table 1.7. Summary of average prevalence for FURD and each pathogen target both reported and calculated true prevalence with 95% confidence interval.

	RP		TP	
	$\bar{x}$	95% CI	$\bar{x}$	95% CI
FURD	26.13	(25.72, 26.53)	NP	NP
FHV	29.09	(27.76, 30.42)	36.85	(35.15, 38.56)
FCV	25.49	(24.62, 26.26)	NP	NP
C. felis	7.64	(6.70, 8.58)	7.71	(6.76, 8.66)
B. bronchiseptica	11.35	(10.91, 11.80)	63.46	(60.03, 66.89)
M. felis	21.68	(21.34, 22.01)	NP	NP

Abbreviations: RP, reported prevalence; TP, true prevalence; $\bar{x}$, mean; CI, confidence interval; NP, not performed.

FHV prevalence ranged from 0 to 78.2% with a mean reported prevalence of 29.09% and a mean calculated true prevalence of 36.85%. FCV prevalence ranged from 0 to 52.4% with a mean reported prevalence of 25.49%. *C. felis* prevalence ranged from 0 to 35% with a mean reported prevalence of 7.64% and a calculated mean true prevalence of 7.71%. *B. bronchiseptica* prevalence ranged from 2.4 to 22.22% with a mean reported prevalence of 11.35% and a calculated mean true prevalence of 63.46%. *M. felis* prevalence ranged from 14.4 to 25% with a mean reported prevalence of 21.68%.

Overall, FHV and FCV were the most prevalent pathogens detected in shelters with *M. felis* being the next most prevalent. These results for FHV and *M. felis*

correspond with previous reports describing the nature of these pathogens as being endemic within cat populations. This high a prevalence of FCV may also indicate that FCV is endemic within feline shelter populations. The large difference in reported prevalence and true prevalence for *B. bronchiseptica* is explained by the fact that the majority of studies utilized bacterial culture for identification of the pathogen. While bacterial culture is very specific for this pathogen it is poorly sensitive and thus the reported prevalence markedly under-reports the true prevalence of the pathogen. *C. felis* has the lowest prevalence in shelters. This is also consistent with what has been previously published about this pathogen and the fact that it is an obligate intracellular bacteria that requires close contact to be spread would limit it's prevalence within shelter populations unlike the viruses.

Overall, while studies ranged from having one to three critical issues. Critical issues included several studies with selection bias, studies with inter-observer reliability and agreement issues, and the lack of specific definitions for upper respiratory disease as well as positive pathogen detection. Even fewer studies reported internal or external validation of their chosen testing methods and none reported sensitivities and specificities for the assays. While all these critical issues were present, the fact that 3 out of 4 studies had reproducible FURD reported prevalence of around 30% suggest that this may be close to the actual prevalence in shelter populations. The pathogens all had much wider ranges and variations in reporting detection. This can be due to the multiple different testing platforms used, the time of sampling of the population, and whether selection bias occurred and which portion of the population was selected as clinically ill animals would be expected to be shedding higher viral and bacterial loads.

This review demonstrates how important it is to not only report validation of assay platforms used in studies and the methodology for study population selection but also the need to assess the body of work on a subject and address issues that have occurred previously in order to improve the quality of future research.

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CHAPTER 3: IDENTIFICATION OF DIVERSE AND UNIQUE MYCOPLASMA SPECIES IN THE FELINE UPPER RESPIRATORY TRACT UTILIZING THE PAN-MYCO SYBR REAL-TIME qPCR ASSAY

Modified from a paper to be submitted to *PLOS Pathogens*

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Abstract

Mycoplasma species are rather ubiquitous in nature, traditionally considered to be animal host specific (e.g. *Mycoplasma felis* being associated with respiratory and ocular disease in cats). Recent publications have reported increased detections of multiple atypical *Mycoplasma* species in domestic animals and repeated zoonotic transmissions between humans and domestic animal species suggesting less host specificity than previously appreciated. This study investigated the hypothesis that *Mycoplasma* species occur in the cat at higher levels of diversity than previously appreciated utilizing the unique molecular based Pan-Myco SYBR qPCR assay for the detection of the *Mycoplasma* genus. This assay was applied to 592 feline upper respiratory tract samples from the conjunctiva, nares, and oro-pharyngium of 190 cats. *Mycoplasma* species identification was performed via sequencing and BLAST analysis on PCR amplicons. Overall, 15 *Mycoplasma* species were detected including: *M. gateae/canadense/arginini* cluster, *M. canis*, *M. hyorhinis*, *M. alkalescens*, *M. cynos*, *M. faucium*, *M. dispar*, *M.*

buccale, *M. spumans*, *M. hominis*, *M. bovis*, *M. bovoculi*, *M. maculosum/leopharyngis* cluster, 2 new unknown species, and *Acholeplasma laidlawii*. Twelve of these *Mycoplasma* species were previously unreported in cats and 2 of these previously unreported *Mycoplasma* species are known zoonotic species with *M. canis* and *M. bovis* both being transmitted to and causing disease in humans. Of the 15 *Mycoplasma* species detected in cats in this study, 7 are known to be zoonotic species causing disease in humans.

Introduction

Mycoplasma species are prokaryotic organisms from the class *Mollicutes* which lack a cell wall and survive on host mucosal surfaces where they can act as primary pathogens, secondary pathogens, or commensals. While *Mycoplasma* species may reside in more than one host, their persistence and correlation with disease is considered to be host specific [1]. Several host specific *Mycoplasma* species are associated respiratory disease syndromes including *Mycoplasma pneumonia* in humans [2], *Mycoplasma pulmonis* in mice and rats [3], *Mycoplasma cynos* in domestic dogs [4], *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides* Small Colony in cattle [1], *Mycoplasma capricolum* subspecies *capripneumoniae* in small ruminants [1], and *Mycoplasma hyopneumonia* in pigs [5].

In domestic cats *Mycoplasma felis* [6-8], *Mycoplasma gateae* [6-9], *Mycoplasma argininei* [10,11], and *Mycoplasma feliminutum* [8,12] are commonly isolated, through culture and detected via molecular methods, from the upper respiratory tracts of cats with and without Feline Upper Respiratory Disease (FURD). *Mycoplasma felis*, *M. gateae*,

and *M. feliminutum* are considered host specific. Of these, *Mycoplasma felis* is considered the most pathogenic species in domestic cats and the etiology of conjunctivitis and FURD [13,14]. Experimentally these diseases have been reproduced through ocular conjunctiva and nasal challenge[14]. However, *Mycoplasma felis* has also been detected through culture and isolation from the mucosal surfaces of clinically healthy cats most likely representing some form of a carrier state [7,15]. *Mycoplasma gateae*, as characterized through both culture and DNA identification, is considered a commensal in cats [7,9] however investigation into its ability to cause disease in cats has not been determined [7,16]. *Mycoplasma arginine*, as identified by culture, is also considered a commensal in cats, is isolated more often than *M. feliminutum,* and failed to cause disease on its own [11]. *Mycoplasma felis* has been transmitted between cats and humans as a zoonosis resulting in cellulitis and septic arthritis [17,18]. *Mycoplasma arginini* was implicated in zoonotic transmission, in an immune-compromised man who had a history of being employed in a slaughter-house facility, leading to a fatal Mycoplasmal septiciemia [19].

The diversity of Mycoplasma species that have been detected in the feline upper respiratory tract previously, and those methods of detection, are summarized in Table 2.1. *Mycoplasma lipophilum/hyopharyngis*, *M. cynos*, *M. pulmonis*, *M. arthritidis*, *M. gallisepticum* and *Acholeplasma laidlawii* have been detected but are not considered host adapted to cats (Table 2.1). Aside from atypical Mycoplasma species occurring in the cat, the feline host specific species, *M. felis,* is reported as a causative agent of respiratory disease in race-horses [20]. *Mycoplasma pneumoniae*, a human host specific species, has

been documented in a transmission cycle from humans to hamsters and zoonotically back to humans [21] and *Mycoplasma salivarium*, also a human and primate host specific species, has been detected in both horses and swine samples suggesting transmission from humans to these domestic animal species [22,23]. *Mycoplasma bovis*, *Mycoplasma canis*, and *Mycoplasma maculosum* have also been involved in presumptive transmission to humans from animal hosts (zoonosis) [24-26].

The detection of multiple atypical *Mycoplasma* species in the cat, repeated zoonotic transmissions, and various transmissions between domestic species suggests less host specificity than previously appreciated. In the feline upper respiratory tract, the limited number of *Mycoplasma* species detected may be due in large part to detection methods utilized and study sample size [6,7,10,11,27].

Table 2.1. *Mycoplasma* Species Previously Reported in the Feline Upper Respiratory Tract, Their Primary Host, Their Host Specificity, and the Method of Previous Isolation/Detection.

Mycoplasma species	Primary Host	Host Specificity[a]	Detection Method
M. felis[6-8][b]	Felid	Y	Culture, Serology, PCR
M. gateae/canadense[7-10]	Multiple	N	Culture, Serology, PCR
M. feliminutum[8,12]	Felid	Y	Culture, PCR
M. arginini[b][10,11]	Multiple	N	Culture, PCR
M. lipophilum/hyopharyngis[6]	Hominid/Porcids	Y	PCR
M. cynos[6]	Canid	Y	PCR
M. pulmonis[7]	Rodent	Y	Culture, Serology
M. arthritidis[7]	Rodent	Y	Culture, Serology
M. gallisepticum[7]	Poultry	Y	Culture, Serology
Acholeplasma laidlawii[7]	Soil	N	Culture, Serology

[a] Specificity for the primary host as indicated by 'N' to demonstrate that the *Mycoplasma* species is not considered host specific and 'Y' for *Mycoplasma* species that are considered host specific.
[b] *Mycoplasma* species which have previously reported zoonotic transmissions.

Traditionally, *Mycoplasma* species were detected via cultivation in tissue culture and this technique can be sensitive for the isolation of some *Mycoplasma* species; however, in general culture has been found a poor and inconsistent method for isolating and identifying these organisms. The disadvantages of tissue cultivation include the large number of *Mycoplasma* species that: are uncultivable by current methodologies, have similar colony morphology, grow in mixed colonies [28] , and/or require an extended length of time for colony growth which allows for overgrowth of other bacteria.

Alternately, molecular methods such as conventional Polymerase Chain Reactions (PCR) and real-time PCR (qPCR) assays have been developed for the specific detection of *Mycoplasma felis* [29,30], *M. bovis* [31], *M. hyopneumonia* [32], and others. Initially conventional PCR offered more rapid and specific detection than traditional culture; however this method suffered from sensitivity issues and the need for labor and time consuming electrophoresis for detection of positives. Advantages of qPCR include: being more rapid, sensitivity, and specific based on fluorescent probes used to detect positives in real-time. With the improved sensitivity and specificity of the probe based assay comes disadvantages which include the identification of missing subspecies with gene mutations and pathogen evolution events. The qPCR assays also have limited application in detection of atypical *Mycoplasma* species due to their high level of specificity. This restricts their use in studies designed to investigate the diversity of *Mycoplasma* species occurring in a host.

This study utilized a unique molecular based SYBR GREEN qPCR assay for the detection of the *Mycoplasma* genus in the feline host to investigate the hypothesis that

Mycoplasma species occur in the cat at a higher level of diversity than previously appreciated. Reported here is the detection of 12 *Mycoplasma* species that had not been previously reported in the cat using the Pan-Myco SYBR qPCR assay. Two of these species were previously reported to be zoonotic and 2 were unknown species. Of the 15 *Mycoplasma* species detected in cats overall in this study 7 were reported zoonotic agents.

Materials and Methods

Mycoplasma Strains and Colony Forming Unit Determination

Mycoplasma felis strain 23391and *Mycoplasma gateae* strain 23392 (American Tissue Culture Collection, Manassas, VA) were reconstituted from lyophilized culture stocks in Friis Broth (Kindly provided by Dr. Ricardo Rosenbusch, Iowa State University, Ames, IA) and *Mycoplasma bovis* strain 25025(American Tissue Culture Collection, Manassas, VA) was reconstituted in Brain Heart Infusion Broth(BBL, Becton, Dickerson and Company, Sparks, MD). Concentrated stocks were stored at -80^{o}C in 40% glycerol stocks and quantitated by colony forming unit determination on Mycoplasma agar (California, Davis).

Clinical Feline Samples

Five hundred and ninety two samples were collected from cats residing in 5 different animal shelters and visiting the Lloyd Veterinary Medical Center. At the time of sampling all cats were evaluated for clinical signs of FURD including conjunctivitis, ocular and nasal discharge, sneezing, and oral stomatitis and faucitis and documented as

having the presence or absence of FURD. Conjunctival, nasal, and deep oropharynx samples were collected using dry sterile nylon flocculated swabs placed into an Amies medium collection system (Copan ESwab 480C and 481C, Copan Diagnostics Inc, CA) and chilled for transport. At the laboratory samples were stored at -80^{o}C until processing. The sampling of cats for this experiment was approved by the Iowa State University Institutional Animal Care and Use Committee.

Nucleic Acid Extraction

Nucleic acid extraction was performed using a commercial spin column extraction kit (DNeasy Blood & Tissue Kit and Quick-Start Protocol, QIAGEN, Valencia, CA). All stocks and clinical samples were defrosted at 4^{o}C for 24 hours and mixed thoroughly via pulse vortex prior to extraction. Procedures followed the kit protocol for non-nucleated blood, with the following modifications: 100ul of sample was used during lysis and incubated for 5 minutes at 56^{o}C, during the final wash samples were centrifuged for 6 minutes at16.3 g force, and nucleic acid was eluted into 100ul of the provided buffer preheated to 56^{o}C. Each extraction of clinical samples included a *M. felis* positive and a phosphate buffered saline (HyClone, Thermo Scientific, Logan, UT) negative control.

Pan-Myco SYBR qPCR assay

Pan-Myco SYBR qPCR was performed for *Mycoplasma* species with the forward primer, MYCP 3a, 5'-CATATGTTCTTTGAAAACTG-3', and the reverse primer, MYCP 4, 5'-GCATCCACCAAAAACTCT-3'. These primer sets were originally developed for *M. bovis* detection using conventional nested PCR [31] and modified for

the Pan-Myco SYBR GREEN PCR[40]. For the qPCR, a reaction volume of 12uL comprised of 2X PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD), forward and reverse primers at .208 uM (Integrated DNA Technologies, Coralville, IA), 2ul of extracted nucleic acid, and PCR-grade H_2O was utilized. Thermocycling conditions performed on a real-time PCR system (Bio-Rad Laboratories, Hercules, CA) were 95^{o}C for 2 min, followed by 40 cycles of 94^{o}C for 30 sec, 55^{o}C for 2 min, and 72^{o}C for 60sec with fluorescence read in the FAM channel during annealing and extension (Figure 1.1). qPCR was immediately followed by melt analysis which included stepwise heating of the block from 55^{o}C to 85^{o}C progressing at an increment of 0.1^{o}C per 10 sec with fluorescence read at each increment. Melt analysis was preceded by the 95^{o}C for 15sec, and 55^{o}C for 6 sec. Melt analysis was used to generate a dissociation curve and determination of the amplicon melting temperature (Tm) (Figure 1.2 and 1.3).

Assay Limits of Detection and PCR Processing of Clinical Sample

Serial dilutions in triplicate of stock *M. felis* and *M. gateae* DNA were run for assay validation, limits of detection, and determination of melt temperature on the Pan-Myco SYBR qPCR. Clinical samples were run in duplicate wells and each plate contained both a stock *M. felis* positive control and a PCR-grade H_2O negative control. Samples which crossed the amplification threshold (Ct) and had a Tm between 68-75^{o}C are positive for *Mycoplasma* species.

***Mycoplasma* species detected by the Pan-Myco SYBR qPCR**

Definitive identification of Pan -Myco SYBR qPCR generated amplicons was performed through direct Sanger sequencing (DNA Sequencing Facility, Iowa State

Figure 1.1. Amplification Curve of the Three Positive Control *Mycoplasma* Species. The Pan-Myco PCR primer set binds to a conserved region of the *Mycoplasma* genome flanking species identifying sequences. PCR generates double stranded DNA (dsDNA) with bound SYBR GREEN dye, rising fluorescence is detected by the thermocycler and demonstrated here with *Mycoplasma felis*(solid line), *Mycoplasma gateae*(circles), and *Mycoplasma bovis* (triangles).

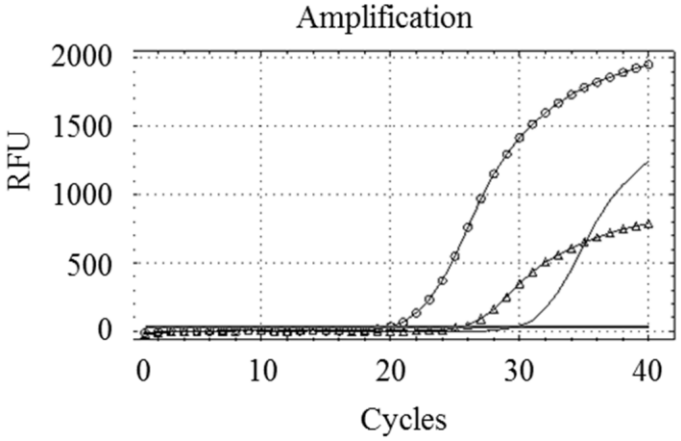
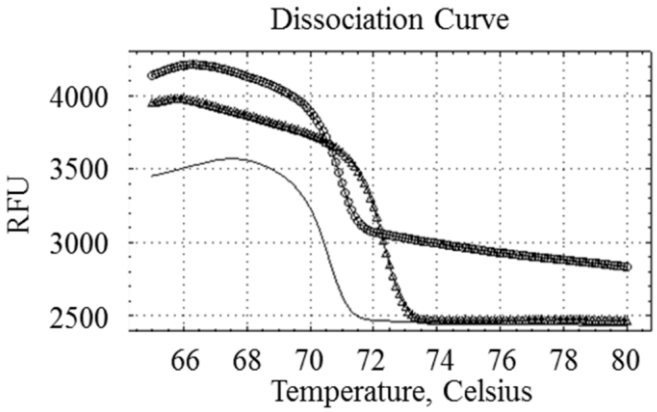


Figure 1.2. Dissociation Curve of the Three Positive Control *Mycoplasma* Species. Incremental increasing in temperature causes the melting of the dsDNA into single stranded DNA (ssDNA) and results in a dissociation curve demonstrated here with *Mycoplasma felis* (solid line), *Mycoplasma gateae* (circles), and *Mycoplasma bovis* (triangles).



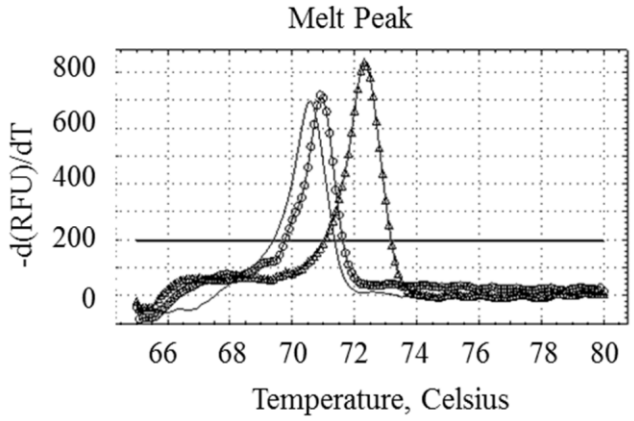


Figure 1.3. Determination of Melting Temperature for the Three Positive Control *Mycoplasma* Species. Incremental increases in temperature causes the DNA binding dye to be released. The specific temperature is determined by changes in fluorescence in relation to time. Melt temperature (Tm) is dependent on amplicon size and GC content and differs between *Mycoplasma* species as demonstrated here with *Mycoplasma felis* (solid line), *Mycoplasma gateae* (circles), and *Mycoplasma bovis* (triangles).

University, Ames, IA). Samples with a single amplicon were sent for sequencing with forward Pan-Myco SYBR qPCR primers. Samples with 2 or more *Mycoplasma* species present were visualized and separated with gel electrophoresis in 2% low melt agarose (Lowmelt, Bio-Rad Laboratories, Hercules, CA). Each gel was pre-stained with nucleic acid gel stain (Gel Star Nucleic Acid Gel Stain, Lonza, Rockland, ME) and included a well of 100bp DNA Ladder (Invitrogen Life Technologies Co, Carlsbad, CA) , *M. felis* positive control, and negative control. A 10X BlueJuice loading dye (Invitrogen Life Technologies Co, Carlsbad, CA) was used and electrophoresis was performed at 120 volts for 60 minutes. Amplicons between 50 and 300 base pairs were excised from the gel, purified using a commercial kit for gel purification (QIAquick Gel Extraction Kit and the Quick-StartProtocol, QIAGEN, Valencia, CA), and the resulting DNA extraction was sent for sequencing as previously described.

The resulting amplicon sequence was subjected to BLAST, and alignments with a high level of query coverage (greater than 80%) and maximum identities (greater than 95%) with a known referenced or commercially available *Mycoplasma* species in GenBank served as definitive species identification when possible.

Results

Validation and Limits of Detection of Pan-Myco SYBR qPCR for Stock *Mycoplasma* Species

Validation of the Pan-Myco SYBR qPCR assay was performed using pure culture stock *Mycoplasma felis*, *Mycoplasma gateae*, and *Mycoplasma bovis* (American Tissue

Culture Collection, Manassas, VA). The standard curve for *M. gateae* had a R^2 of 0.996 and an amplification efficiency of 78% and a Tm of 70.9°C (SE=0.18) (Figure 1.4). The standard curve for *M. felis* was determined to have an R^2 of 0.996 and an amplification efficiency of 121% (data not shown). The Pan-Myco PCR Assay successfully amplified all three stock Mycoplasma species and could differentiate species based on the Tm of the qPCR amplicon (Figure1.1, 1.2, and 1.3). The mean Tm for *M. felis* was determined to be 70.4°C (SE=0.14) and the mean Tm for *M. bovis* was 72.3°C (SE =0.30). The assay primers amplified a 16s area corresponding to 180 base pairs for *M. gateae*, 200 base pairs for *M. felis*, and 220 base pairs for the *M. bovis* (Figure 1.5). The limits of detection for the Pan-Myco SYBR qPCR for *M. gateae* and *M. felis* were equivalent to 1 CFU. The Pan-Myco SYBR qPCR limits of detection for genome equivalents based on taqman qPCR[29] in droplet digital PCR[33] was .3 genome equivalents for *M. felis*.

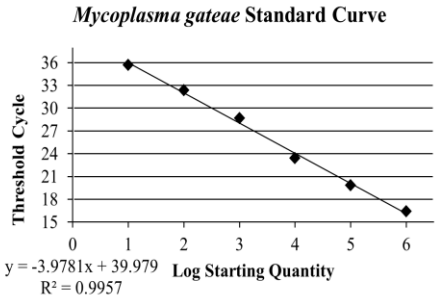


Figure 1.4. *Mycoplasma gateae* Standard Curve on the Pan-Myco SYBR qPCR Assay.
This graph demonstrates the standard curve for the *M. gateae* pure culture control which was utilized to validate the Pan-Myco SYBR qPCR assay. Standard curve was created via the running of a serial dilution set of the standard DNA in triplicate. The standard curve demonstrates an R^2 of 0.996 and an amplification efficiency of 78%.

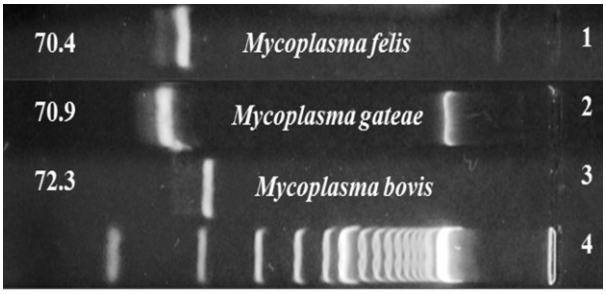


Figure1. 5. Agarose Gel Electrophoresis of Pan-Myco SYBR qPCR Amplicons for Control Species.
This photographs of a agarose gel electrophoresis shows lane 1 containing a 200 bp amplicon for stock *Mycoplasma felis* species with a Tm of 70.4, lane 2 containing a 180 bp amplicon for stock *Mycoplasma gateae* species with a Tm of 70.9, lane 3 with a 220 bp amplicon for stock *Mycoplasma bovis* species with a Tm of 72.3, and lane 4 containing a 100bp molecular marker.

Mycoplasma Species Detected in Clinical Samples

Fifteen species of *Mycoplasma* and 1 species of *Acholeplasma* were detected in feline clinical samples utilizing the Pan-Myco SYBR qPCR assay. These include 3 species previously reported in cats (*Mycoplasma arginini/canadense/gateae* cluster, *Mycoplasma leopharyngis/maculosum* cluster, and *Mycoplasma cynos*), 10 species previously unreported in cats (*Mycoplasma buccale*, *Mycoplasma facium*, *Mycoplasma dispar*, *Mycoplasma hyorhinis*, *Mycoplasma bovis*, *Mycoplasma hominis*, *Mycoplasma spumans*, *Mycoplasma alkalescens*, *Mycoplasma canis, Mycoplasma bovoculi),* and two unknown *Mycoplasma* species. The two unknown species had sequences that attained low identity matches with GenBank (FR799684.1 and FR799683.1) and were stated as unculturable *Mycoplasma* species from the intestines of fish. Table 2.2 provides a list of *Mycoplasma* species detected with the corresponding GenBank accession numbers that were used for definitive identification of the species based upon percent homology (expressed as percent query coverage and percent maximum identity). Amplicon size as determined by electrophoresis and Tm determined by dissociation curve analysis following Pan-Myco SYBR qPCR are also reported as well as the anatomic location of detection and the percent positive samples.

Mycoplasma arginini/canadense/gateae cluster was the most commonly detected species and occurred in 7.77% of the samples, of which 37 of the 46 positive sequences were obtained from the oral cavity. This *Mycoplasma* species (*M. arginini/canadense/gateae*) is reported as a cluster due to the limited variability of the 16s intergenic region of the *Mycoplasma* genus that the Pan-Myco SYBR qPCR assay

amplifies. Similarly, *Mycoplasma leopharyngis* and *Mycoplasma maculosum* had a 99% identity in this amplified region and are also reported as a cluster. The *Mycoplasma leopharygnis/maculosum* cluster occurred in 1.01% of the samples and *Mycoplasma spumans* was detected in 2.03% of the samples. Additionally, *Acholeplasma laidlawii* was detected with the Pan-Myco SYBR qPCR assay. While *A. laidlawii* is not a *Mycoplasma* species, it is a member of the class Mollicutes and the amplicon generated had 24.2% homology with the 16S ribosomal RNA of the *M. felis* amplicon. Other *Mycoplasma* species were considered rare detections and occurred in less than 1% of the samples. These comprised the majority of the species detected (13 out of 15). Of the 592 samples screened with the Pan-Myco SYBR qPCR assay 12.3% had 1 or more *Mycoplasma* species detected. The melt temperatures determined by the SYBR GREEN ranged from 69.5^{o}C to 73.5^{o}C some of which overlapped with multiple *Mycoplasma* species while others were unique. While the Pan-Myco SYBR qPCR assay allowed for the detection of atypical and rare *Mycoplasma* species events in cats it also allowed for the detection of more than one *Mycoplasma* species in a single sample.

Ten of the samples collected included concurrent detection of 2 or more *Mycoplasma* species. Table 2.3 demonstrates the anatomical site of origin, whether FURD was present or absent in the cat the sample was obtained from, and the Mycoplasma species that were detected in the sample. In this table, *Mycoplasma gateae* and *Mycoplasma leopharyngis* are used to represent their respective clusters. Three samples contained 3 *Mycoplasma* species, while 7 samples contained 2 *Mycoplasma* species. Of the species involved in co-detection, 60% of the samples contained the

Table 2.2. *Mycoplasma* Species Detected in Feline Clinical Samples Using the Pan-Myco SYBR qPCR Assay.

Mycoplasma species	GenBank[a]	% Homology		Amplicon size[d]	Tm[e]	Anatomic location			% Positive cases[f]
		A[b]	B[c]			Oral	Nasal	Ocular	
M. arginini	JN935883.1								
M. canadense	AY800341.1	88-99	94-100	180 BP	70.7-72.7	37	3	6	7.77
M. gateae	AY973562.1								
M. leopharyngis	AY762644.1	81-99	84-97	220 BP	71.8	2	1	3	1.01
M. maculosum	AF443610.1								
M. spumans	AF538684.1	92-100	92-100	200 BP	71.7-72.5	2	1	9	2.03
M. alkalescens	AY816348.1	96-99	98-99	ND	71.4-72.2	0	1	2	<1
M. canis	AF443605.1	96-99	97-98	ND	71.9-72.1	1	0	2	<1
M. cynos	AF538682.1	95-97	88-99	200 BP	72.4	1	0	2	<1
M. buccale	AY796064.1	94	92	190 BP	71.9	0	1	0	<1
M. faucium	AY800342.1	100	100	ND	72.2	0	0	1	<1
M. dispar	DQ840510.1	99	97	ND	69.5	0	0	1	<1
M. hyorhinis	CP003914.1	98	99	220 BP	71.5-72.5	1	0	1	<1
M. bovis	AY566217.1	98-99	99-100	250 BP	72.6-73.9	1	0	2	<1
M. hominis	FP236530.1	95	100	160 BP	71.3	1	0	0	<1
M. bovoculi	AY785380.1	99	99	ND	71.7	0	0	1	<1
Uncultured sp.	FR799684.1	89	82	200 BP	73.2	0	1	0	<1
Uncultured sp.	FR799683.1	91	93	ND	73.5	0	0	1	<1
A. laidlawii	CP000896.1	83	100	100 BP	71.8	0	0	1	<1

[a] GenBank Accession number identified through BLAST analysis of amplicon sequences

[b] Percent Query Coverage (>80% considered definitive *Mycoplasma* detection), ranges reported span the query coverage from multiple positives

Table 2.2 footnotes continued…
[c] Percent Maximum Identity (>95% considered definitive *Mycoplasma* detection), ranges reported span the maximum identity from multiple positives
[d] Amplicon size in base pairs acquired through gel electrophoresis when performed, ND = Not Done
[e] Tm determined by post-qPCR dissociation curves, the ranges are reported for multiple positives
[f] Percent of occurrences of the *Mycoplasma* species identified out of 592 samples.

Table 2.3. Detection of Multiple *Mycoplasma* Species Concurrently in Individual Cat Samples by the Pan-Myco SYBR qPCR Assay.

Sample type	FURD status[a]	*Mycoplasma* species detected			No. of *Mycoplasma* species
Nasal	P	*M. alkalescense*	*M. buccale*	*M. leopharygis*[b]	3
Oral	P	*M. gateae*[b]	*M. leopharygis*	*M. spumans*	3
Ocular	P	*M. gateae*	*M. spumans*	*M. bovis*	3
Ocular	P	*M. dispar*	*M. hyorhinis*		2
Ocular	P	*M. gateae*	*M. maculosum*		2
Oral	P	*M. gateae*	*M. leopharygis*		2
Ocular	A	*M. leopharygis*	*M. spumans*		2
Ocular	A	*M. gateae*	*A. laidlawii*		2
Oral	A	*M. gateae*	*M. hominis*		2

[a] Feline upper respiratory disease P = present, A = absent
[b] Denotes that these *Mycoplasma* species are members of a previously discussed cluster; for *M. leopharyngis* this includes *M. maculosum*, for *M. gateae* this cluster includes *M. arginini* and *M. canadense*

Mycoplasma gateae cluster. All of the samples with 3 *Mycoplasma* species detected came from cats with FURD, while 57.14% of the samples with dual detections came from cats with FURD. Ocular conjunctival swabs comprised 60% of the samples, while 30% were deep oro-pharyngeal swabs, and a single swab came from the nasal planum. Along with reporting the co-occurrence of *Mycoplasma* species in a single anatomical location, the Pan-Myco SYBR qPCR in conjunction with holistic upper respiratory sampling allowed for the reporting of the ecology of upper respiratory *Mycoplasma* species in individual cats.

Fourteen of the 190 cats tested (7.37%) had multiple *Mycoplasma* species concurrently detected from their collection of samples. In Table 2.4 the anatomical distribution of *Mycoplasma* detection, the occurrence in individual cats, whether those cats had FURD, and the species present samples from those cats are demonstrated. Again, *Mycoplasma gateae* and *Mycoplasma leopharyngis* are used to represent their respective clusters. Three of the 14 cats (21.4%) had 3 Mycoplasma species detected from their collection of samples. 21.4% of the cats with multiple Mycoplasma species detected concurrently in multiple anatomical locations had FURD. All but 1 of the cats (92.86%) had the *Mycoplasma gateae* cluster detected in a sampling location.

Table 2.4. Distribution and Speciation of Multiple *Mycoplasma* Species Occurring Concurrently in Individual Felids.

Samples	Occurrence[a]	% with FURD	*Mycoplasma* species present		
Oral/Ocular/Nasal	1	0	*M. gateae*[b]	*M. spumans*	*M. alkalescens*
Ocular/Nasal	1	0	*M. gateae*	*M. leopharygis*[b]	*M. spumans*
Oral/Ocular	1	100	*M. gateae*	*M. dispar*	*M. hyorhinis*
Oral/Ocular	2	0	*M. gateae*	*M. cynos*	
Oral/Ocular	1	0	*M. gateae*	*A. laidlawii*	
Ocular/Ocular	1	100	*M. gateae*	*M. maculosum*	
Oral/Ocular	1	0	*M. gateae*	*M. canis*	
Oral/Ocular	3	0	*M. gateae*	*M. spumans*	
Oral/Ocular/Nasal	2	50	*M. gateae*	*Uncultured sp.*	
Ocular/Ocular	1	0	*M. spumans*	*M. cynos*	

[a] Occurrence of this combination in individual cats
[b] Denotes that these *Mycoplasma* species are members of a previously discussed cluster; for *M. leopharyngis* this includes *M. maculosum*, for *M. gateae* this cluster includes *M. arginini* and *M. canadense*

Discussion

Although *Mycoplasma* species have been traditionally considered host specific pathogens, the increasing isolation, through culture methods, and detection, through molecular methods, of multiple atypical *Mycoplasma* species in domestic animals and repeated zoonotic transmissions suggest less host specificity than previously appreciated. This study investigated the hypothesis that *Mycoplasma* species occur in the cat at a higher level of diversity than previously detected. This hypothesis was tested utilizing the unique molecular based Pan-Myco SYBR qPCR assay for the detection of the *Mycoplasma* genus. Application of this assay to 592 feline upper respiratory tract samples from 190 cats resulted in the identification of 15 *Mycoplasma* species including: *M. gateae/canadense/arginini* cluster, *M. canis*, *M. hyorhinis*, *M. alkalescens*, *M. cynos*,

M. faucium, *M. dispar*, *M. buccale*, *M. spumans*, *M. hominis*, *M. bovis*, *M. bovoculi*, *M. maculosum/leopharyngis* cluster, 2 new unknown species, and *Acholeplasma laidlawii*. The *Mycoplasma* species detected in this study and in previous studies are summarized in Table 2.5. This table includes whether detection in this study was a novel detection in cats, what the principal host for this *Mycoplasma* species is, whether the *Mycoplasma* is pathogenic in that principal host, and whether it has been previously reported as a zoonosis. In total, 71 (12%) of the 592 samples tested had *Mycoplasma* species detected in them with 26 of those detections (37%) being novel in the cat.

Overall, 15 *Mycoplasma* species were detected in the feline samples in this study with 12 (80%) of the *Mycoplasma* species detected being previously unreported in the feline upper respiratory tract. These include: *M. spumans*, *M. canis*, *M. alkalescens*, *M. dispar*, *M. bovis*, *M. bovoculi*, *M. buccale*, *M. faucium*, *M. hominis*, *M. hyorhinis* and two new unknown species. The two new unknown species had low homology to GenBank accessions suggesting that they are actually separate new species. Further work is necessary to characterize these. The most frequently detected atypical species were *M. spumans*, *M. canis*, *M. alkalescens*, and *M. bovis*. The majority of *Mycoplasma* species detected in this study were novel identifications in the cat demonstrating that the diversity of *Mycoplasma* species is much greater than previously appreciated. These novel detections in the feline host suggest that cats are capable of harboring a much broader range of *Mycoplasma* species with unknown consequences to the cat itself. Many of these *Mycoplasma* species detected are considered pathogenic in other domestic animal species and in humans. This finding is not completely unique as multiple studies using qPCR and

Table 2.5. Summary of the *Mycoplasma* Species Detected in this Study as well as Those Previously Reported in the Feline Upper Respiratory Tract.

Mycoplasma species[a]	Novel detection?[c]	Principal Host	Pathogenic?[d]	Reported zoonosis[e]
M. gateae[b]	N	Multiple	Y/N	Y
A. laidlawii	N	Multiple	Y/N	NA
M. leopharyngis[b]	N	Canid/Felid	N	Y
M. spumans	Y	Canid	Y	N
M. canis	Y	Canid	N	Y
M. cynos	N	Canid	Y	N
M. alkalescens	Y	Cattle	Y	N
M. dispar	Y	Cattle	Y	N
M. bovis	Y	Cattle	Y	Y
M. bovoculi	Y	Cattle	Y	N
M. buccale	Y	Human	Y	NA
M. faucium	Y	Human	Y	NA
M. hominis	Y	Human	Y	NA
M. hyorhinis	Y	Swine	Y	N
M. lipophilum[b]	ND	Human, Swine	N	NA
M. pulmonis	ND	Rodent	Y	Y
M. arthritidis	ND	Rodent	Y	Y
M. gallisepticum	ND	Poultry	Y	N
M. feliminutum	ND	Felid	N	N
M. felis	ND	Felid, Equid	Y	Y

[a] *Mycoplasma* species that are underlined are those species detected in this study
[b] Denotes that these *Mycoplasma* species are members of a previously discussed cluster; for *M. leopharyngis* this includes *M. maculosum*, for *M. gateae* this cluster includes *M. arginini* and *M. canadense;* for *M. lipophilum* this includes *M. hyopharyngis*
[c] Novel detection of *Mycoplasma* species in cats, N= No, ND= Not Detected, Y = Yes
[d] Is this *Mycoplasma* species considered pathogenic in its host, Y = Yes, No = No, Y/N = Depends upon the host and host conditions
[e] Previously reported as zoonotic, Y = Yes, N = No, NA = Not Applicable

other various high throughout sequencing methodologies and platforms [34] are discovering an ever increasing number of bacterial pathogens in humans that, similar to this study, were uncultivable yet the dominate bacterial pathogen genome present in diseased tissue and disease conditions such as respiratory diseases [35].

The presence of previously undetected Mycoplasma species in cats that are significant pathogens in humans or other domestic animal species also suggests that cats can become infected with and harbor, apparently without disease, these Mycoplasma species with currently unknown consequences to both the cats and the other species. *M. spumans* is a pathogenic *Mycoplasma* species in the dog causing arthritis and pneumonia, while *M. canis* is a commensal species of the canine upper respiratory tract [36]. *M. alkalescens*, *M. bovis*, and *M. dispar* are both bovine pathogenic *Mycoplasma* species causing mastitis, respiratory disease, and joint disease [1]. *M. bovoculi* is associated with ocular disease in cattle and may contribute to Bovine Infectious Keratoconjuncitivis [1]. Both *M. bovoculi* and *M. bovis* are pathogens of considerable concern in the cattle industry. *M. hyorhinis* causes arthritis and pneumonia in swine [1] and *M. buccale*, *M. faucium*, and *M. hominis*, are all considered human host specific *Mycoplasma* species [37]. *M. buccale* and *M. faucium* are rarely identified and are of concern in immunocompromised humans. *M. hominis* has been associated with pyelonephritis and pelvic inflammatory disease in humans [37].

These findings may well be important and especially relevant to public health when the close relationship between cats, their human owners, and other animal species are considered in disease outbreaks. Not only does this data suggest that cats are capable of harboring multiple *Mycoplasma* species considered human host specific pathogens but many of the *Mycoplasma* species detected in this study have also been previously reported to have zoonotic capabilities and transmissions.

This study detected 4 *Mycoplasma* species in cats that have had previously reported zoonotic transmissions. Of these, 2 *Mycoplasma* species are novel detections in the feline host. These include *M. canis*, which colonized several individuals of a household with one person developing respiratory infection [25] , and *M. bovis*, was isolated from a woman with systemic illness including pneumonia [24]. *M. arginini* was detected in this study as part of the *M. gateae* cluster and is reported to have caused septicemia in a slaughterhouse worker [19] and *M. maculosum* has been reported to have been isolated from the cerebrospinal fluid of a patient with meningitis who was assumed to have contracted the *Mycoplasma* from his dog [26]. In previous studies, *M. felis*, *M. pulmonis*, and *M. arthritidis* have been reported in cats and all also have been reported in zoonotic transmissions. *M. felis* has caused cellulitis after a cat scratch and closed septic arthritis after close contact with cats [17,18]. *M. pulmonis* was found to be colonizing technicians working with research rats [38] and *M. arthritidis* has been isolated from synovial tissue of laboratory workers [39].

The detection of these multiple *Mycoplasma* species in the feline host may suggest that the evolved relationship leading to domestication of cats with humans may have established various adaptations in one or the other species and in some cases with unforeseen isolated disease cases and/or larger public health ramifications, especially for immune-compromised individuals. The rare occurrence of these *Mycoplasma* species in this study and the lack of inclusion of other FURD pathogens prevented analysis of association between the concurrent presence the *Mycoplasma* species and feline disease in this population. It should however be noted that there was a high concurrence between

multiple *Mycoplasma* species detected and FURD. Further investigation is warranted into the possibility of association and causation between rare *Mycoplasma* species being harbored in cats with disease.

The finding of 12 novel *Mycoplasma* detections in cats, 2 of which are new species and 2 which are reported zoonotic species as well as the complete detection of 15 species of *Mycoplasma* many of which are considered host specific pathogens in either humans or other domestic animal species support the hypothesis that that *Mycoplasma* species are not as host specific as previously assumed. The finding of multiple *Mycoplasma* species in a single site in concurrence with FURD also supports a further investigation into the nature of *Mycoplasma* colonization and whether they are pathogenic. This study also demonstrates the necessity sampling both the ocular conjunctiva and oro-pharynx when investigating *Mycoplasma* infection in cats. Finally, the broad diversity of *Mycoplasma* species detected in this study and the concurrent detections would not have been possible without a broad detection tool such as the Pan-Myco SYBR qPCR. The Pan-Myco SYBR qPCR proved to be capable of broad detection of Mycoplasma species of both veterinary and public health importance in the feline host.

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CHAPTER 4: VALIDATION OF A PORTABLE MOLECULAR DIAGNOSTIC DEVICE FOR DETECTION OF PATHOGENS ASSOCIATED WITH FELINE UPPER RESPIRATORY DISEASE IN CATS

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Abstract

As diagnostic tools advance there is a growing number of rapid, effective, field diagnostic tools for the identification of pathogens. A 5 stage pipeline was determined for development and validation of field deployable devices. This present study utilizes pathogens related to Feline Upper Respiratory Disease including Feline Herpesvirus, Feline Calicivirus, *Chlamydophila felis*, *Mycoplasma felis*, and *Bordetella bronchiseptica*, and the candidate field deployable device, POCKIT™. Validation, including limits of detection and exclusivity testing, of the pathogen specific Insulated Isothermal Polymerase Chain Reaction (iiPCR) as reagents on the field deployable device was performed against published reference assays. Thirty of both positive and negative clinical samples and surrogate samples were randomized, blinded, and tested side-by-side

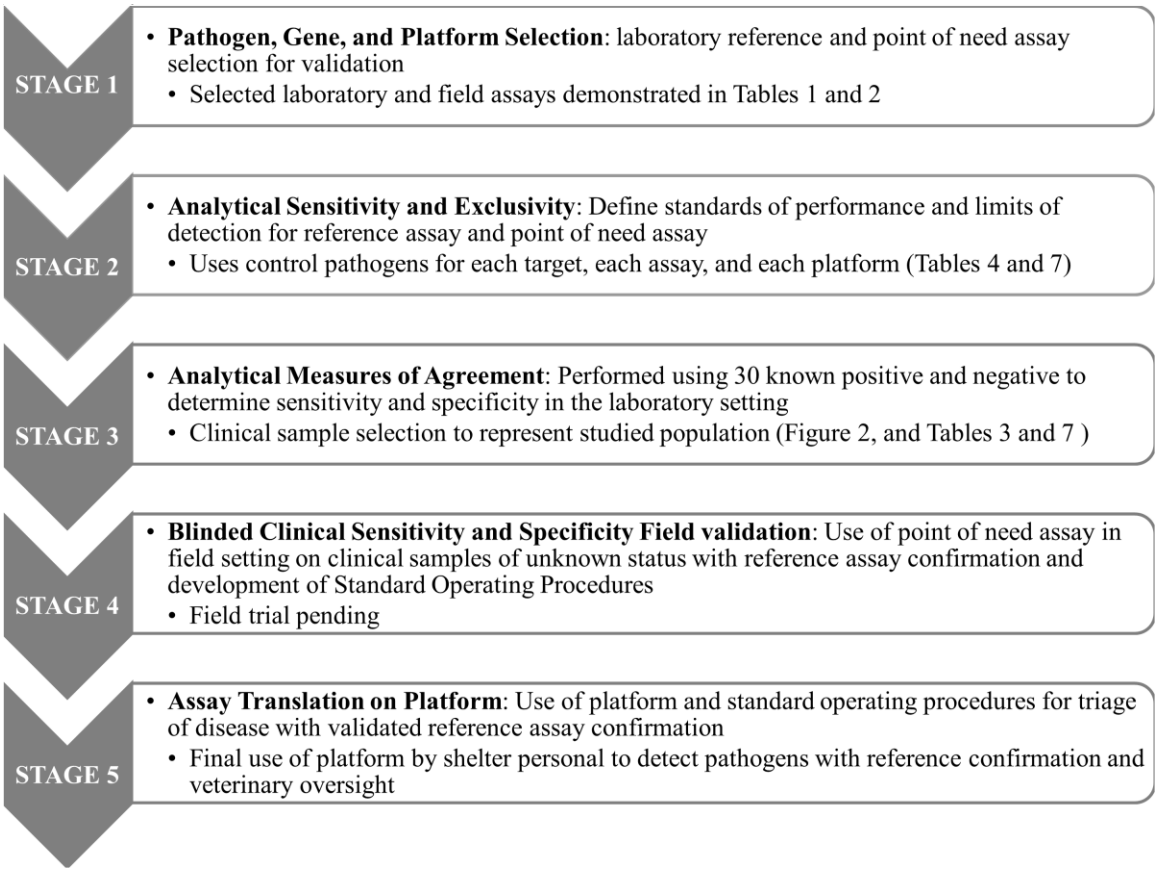
on both platforms for each pathogen. Limits of detection were biologically relevant, were equal to or less than 10 infectious units, and demonstrated near equivalency with clinical samples for all pathogen targets. Exclusivity testing demonstrated the iiPCR to be pathogen and target specific. Sensitivity and specificity for clinical samples ranged from 80%-97% and 93-100% respectively. Kappa values ranging from 0.80- 0.93 demonstrated strong agreement. Results demonstrate exceptional performance of the iiPCR reagents for detection of feline respiratory pathogens in clinical samples. This study demonstrates the effectiveness of the iiPCR for detection of feline pathogens and the use of the stages 1 through 3 of the pipeline for validation of field deployable reagents.

Introduction

There is currently a drive for rapid, effective field diagnostic tools for the identification of pathogens. A five stage validation and translational pipeline for point of need diagnostics was developed and implemented for this purpose (Figure 1). This systematic validation process is to ensure that point of need diagnostics are equivalent to current reference laboratory assays in sensitivity, specificity, exclusivity, and that results are reproducible in both laboratory and field settings. This streamlined validation system also allows for the rapid translation of assays to point of need field devices. The goal of this pipeline is to develop panels of assays that can be run in the field allowing for the early detection and triage of significant diseases. Once a target is detected, the sample would then be sent to a reference diagnostic laboratory for confirmation.

This study illustrates the five stage validation and translational pipeline through its application to Feline Upper Respiratory Disease Complex (FURD) pathogens as proof of concept with a candidate field deployable device. This disease complex is a leading cause of morbidity and mortality in cats, especially cats in group housing such as animal

Figure 2.1. The Point of need reagent development, validation, and translational pipeline. This figure demonstrates the five general stages which can be applied to the rapid development and validation of a point of need reagents and their application in this specific study.



shelters and catteries[6]. In a survey of 157 shelters in the Western United States, FURD was one of the top three diseases of concern reported by shelter workers[15]. FURD can be devastating in shelter environments as the pathogens spread quickly and cats enter

shelters with unknown vaccination and health statuses[6]. Additionally, cats in a stress inducing environments become immune compromised[6]. While these factors can be affected through management practices such as isolation and vaccination of cats, the ability to properly diagnose and triage animals with FURD is essential to prevent the spread of disease, morbidity, and mortality.

Pathogens that contribute to the development of disease and are targets for detection and identification include Feline Herpesvirus (FHV), Feline Calicivirus (FCV), *Bordetella bronchiseptica (B. bronchiseptica)*, *Chlamydophila felis (C. felis)*, and *Mycoplasma felis (M. felis)*[7,8,13,16,17]. Proper identification of the etiological agents' specific to a FURD outbreak in a shelter environment allows for the targeted management and treatment. Currently pathogen detection is performed using molecular techniques at reference laboratories. The testing itself is expensive and shipping increases the time to results as well as the possibility of damage and loss of the samples. With the increase in veterinary involvement in shelters and the growth of shelter medicine, an in-shelter point of need molecular detection system with equivalent sensitivity and specificity to reference assays would offer both a time and cost efficient means for early detection and triage of FURD for veterinarians while confirmatory testing occurs at reference laboratories.

The candidate device, POCKIT™ is small, stable, portable, and cost-efficient and could serve as a point of need device in the shelter environment for detection of FURD pathogens. This device uses lyophilized real-time compatible enzyme mixtures and oligonucleotides to perform Insulated Isothermal Polymerase Chain Reaction (iiPCR).

The iiPCR utilizes Rayleigh-Benard convection PCR[12] in a capillary tube chamber[3] with thermal baffeling[2] in order to allow for PCR convection and amplification. The iiPCR system utilizes natural thermal convection within a capillary tube, caused by a stationary heat source maintained at a constant heat of 95^{o}C, to drive fluid cycling[2]. The cycling of the reaction contents occurs through a temperature gradient that allows for denaturation, primer annealing, and elongation similar to qPCR. Completion of amplification of the nucleic acid occurs in under an hour. Initial proof of concept research has led to Office International des Epizooties (OIE) validation for detection of White Spot Syndrome Virus infection in *Litopenaeus vannamei* (shrimp)[26].

The objective of this study was to demonstrate the application of this validation pipeline for field deployable tests through the validation of the iiPCR for FURD pathogens. In this study, stages 1 through 3 of the validation pipeline were performed using the iiPCR Taqman reagents for the FURD pathogen targets FHV, FCV, *Bordetella* species (*B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*), *C. felis*, and *M. felis* and reference laboratory assays. The data below provides evidence to demonstrate that the iiPCR is both sensitive and specific for the FURD targets and can serve as a point of need device for detection of pathogens relevant to shelter medicine and feline health.

Materials and Methods

Viruses and Bacteria

M. felis strain 23391[a] was reconstituted from lyophilized culture stocks in Friis Broth[b], *B. bronchiseptica* strain 03127[a] was reconstituted from lyophilized culture stocks

in BHI Broth[c], FHV strain VR-636[a], *C. felis* strain VR-120[a], and FCV strain VR-530[a] were sourced from commercially available culture stocks. All pathogens were stored at -80°C (Table 1).

Colony forming units (CFU) were determined for *M. felis* and *B. bronchiseptica* reference strains through ten-fold serial dilutions spread in triplicate on Mycoplasma agar[d] and Blood agar plates[e] respectively. The plates were observed for growth and colony forming units were counted. The Mean Tissue Culture Infectious Dose (TCID50) for FHV and FCV, and Mean Chicken Embryo Infectious Dose ($CEID_{50}$) for *C. felis* were sourced from the commercial product strain data sheet[e] and extrapolated for DNA/RNA extraction and assay limits of detection.

Nucleic Acid Extraction

Manual nucleic acid extraction was performed using a commercial spin column extraction kit[g]. Procedures followed the kit protocol for non-nucleated blood, with the following modifications: 100µl of sample was used during lysis and incubated for 5 minutes, during the final wash samples were centrifuged for 6 minutes at16,300 x g force, and nucleic acid was eluted into 100µl of the provided buffer preheated to 56°C. Extracted nucleic acid was stored at -80°C. All extractions included pure target stock positive control and PCR-grade H_20 negative control which were tested to confirm extraction success via qPCR.

Real-time PCR Reference Assays

Table 1 includes the previously published primer and probe oligonucleotide sequences utilized for each assay, qPCR gene target, and positive control reference strain. All reference assays reported target gene accept for FHV and *C. felis*, for which the target genes were TK (GeneBank Accession FJ478159.2) and ompA (GeneBank Accession AP006861.1) respectively. *M. felis* qPCR[14] was modified to included primers at 0.3µM concentration each, probe at 0.1µM concentration, and 4 µl of nucleic acid in a 24µl reaction. FHV and *C. felis* qPCR [9] was modified to single-plex 12.5µl reactions with primers and probes at 0.1uM concentration and 2.5µl of nucleic acid. *Bordetella* species qPCR[10] was modified to include primers at 0.2uM concentration each, probe at 0.1µM concentration, and 5µL of nucleic acid in a 25µl reaction. FCV qPCR[1] utilized primers at 0.3µM concentration, probe at 0.2µM concentration, and 5µL of nucleic acid in a 25µl reaction. All reactions utilized a commercially available qPCR mastermix[h].

Thermocycling was performed as described in references with the exception of the FCV assay where modifications included a cDNA synthesis step at 45^{o}C for 10 minutes followed by an initial denaturation step at 95^{o}C for 1 minute and 45 PCR cycles of 95^{o}C for 15 seconds, 56^{o}C for 1 minute, and 72^{o}C for 30 seconds. Each qPCR run included a well of the target control stock nucleic acid positive control and PCR-grade H_2O negative control. Thermocycling was performed on a qPCR instrument[i].

Internal Laboratory Validation of Selected Reference qPCR Assays

Tenfold serial dilutions of extracted standard nucleic acid were prepared in PCR-grade H_2O for each reference strain and aliquots were made and frozen at -20^{o}C. Dilutions of the standard nucleic acid were tested in triplicate in 3 scientific replicates for a total of 9 replicates. Standard curves using the 9 replicates were generated for each assay. Standard curves were utilized to determine amplification efficiency and threshold cycle (Ct) for each assay. Once threshold limits were determined for each reference assay, all 187 clinical samples were run for each pathogen target in duplicate. Reference strain positive controls and PCR-grade H_2O negative controls were included and analysis of runs were normalized. Samples with Cts below the threshold limit were considered 'positive'. Samples with no Ct were considered 'not detected'. Samples with Cts above the threshold limit were considered 'suspect' and excluded from the study as they could not be classified as positive or not detected.

Insulated Isothermal PCR Reagents

Designed on the basis of the probe hydrolysis-based method described previously [19], the iiPCR reactions included primers and FAM-labeled hydrolysis probes specific for each target. Nucleotide sequences available in the GenBank database were aligned to identify conserved regions to design target-specific primers and probes for the iiRT-PCR reagents. For *B. bronchiseptica* detection, the *Bordetella* species iiPCR reagent was used. This reaction targeted the fimA gene and could detect three *Bordetella* species, namely *B. bronchiseptica, B. pertussis,* and *B. parapertussis*.

Table 3.1. The target pathogen, gene target, primer/probe oligonucleotide sequences[m], and the reference control for each pathogen.

Pathogen	Target Gene	Primer/Probe sets (5'→ 3')		Region	BP	Positive Control[a]
FHV[12]	TK	Forward	GGACAGCATAAAAGCGATTG	66291-6631	75	VR-636
		Reverse	AACGTGAACAACGACGCAG	66365-66347		
		CY5 Probe	AATTCCAGCCCGGAGCCTCAAT	66319-66340		
FCV[1]	ORF1	Forward	GTAAAAGAAATTTGAGACAAT	1-21	120	VR-530
		Reverse	TACTGAAGWTCGCGYCT	120-104		
		FAM Probe	CAAACTCTGAGCTTCGTGCTTAAA	26-49		
Bordetella species[13]	FimA	Forward	ACTATACGTCGGGAAATCTGTTTG	838163-838140	81	03127
		Reverse	CGTTGTCGGCTTTCGTCTG	838083-838101		
		FAM Probe	CGGGCCGATAGTCAGGGCGTAG	838134-838113		
C. felis[12]	ompA	Forward	GAACTGCAAGCAACACCACTG	1107246-1107266	77	VR-120
		Reverse	CCATTCGGCATCTTGAAGATG	1107322-1107302		
		FAM Probe	CGCTGCCGACAGATCAAATTTTGCC	1107268-1107292		
M. felis[19]	tuf	Forward	TAAATTAGCTCTTGATGGTGTTCCT	469-493	100	23391
		Reverse	TTCAAAGTCTTTTTCTGGAGTTTCA	568-544		
		HEX Probe	TGAGAAGAAAAAGTTATGGAATTAA TGGATGCA	497-529		

Components of the iiPCR reaction, including dNTP, Taq DNA polymerase, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT, were lyophilized into two separate tubes, named Primer/Probe and dNTP/Enzyme, for each reagent. Primer/Probe was rehydrated with 50 µL of Reconstitution Buffer and the resulting 50 µL mixture was then used to rehydrate dNTP/Enzyme. 5µL of the nucleic acid target or extracted sample was then added to this 50µL mixture and then 50µL of this total mixture was then transferred to the final reaction tube specific for this platform[j]. The final reaction tube is then spun in a mini centrifuge[j] for 10 seconds. After which the final reaction tubes are placed into the reaction chamber of commercially available platform nucleic acid analyzer[j] and the reaction program including reverse transcription and PCR was begun. Readings of the 520-nm fluorescent signals generated from PCR amplification were collected and converted to "+" (positive) and "-"(negative) readouts by the system[26]. This plus minus detection system uses measurements of fluorescence before and after cycling to determine the ratio of fluorescence and a ratio of greater than 1.5 is reported as a "+". These results are then saved to a secure digital card in a commercially available data management program[k].

Limits of detection and exclusivity testing

LODs were determined in the following manner: the results from the original internal laboratory validation of the reference assays for each pathogen were analyzed and threshold cut off for the assays were determined to be the last dilution level at which all 100% of replicates had amplification above the Ct. Once this level was determined, two dilutions below and above this level as well as the cut-off dilution were run in 4

replicates iiPCR reagent replicates side-by-side with 3 qPCR reference assay replicates. 100% LOD was determined for the iiPCR based on these 4 replicates and is defined as the last replicated to be 100% positive, although in many cases 75% and 50% detection occurred past the 100% LOD occurred.

Exclusivity testing was performed at the 10^4 concentration of nucleic acid above the 100% reaction LOD for each iiPCR reagent and qPCR assay using FHV, FCV, *B. bronchiseptica*, *C. felis*, and *M. felis* nucleic acids. The *M. felis* iiPCR reagent was additionally tested with *M. bovis* strain 25025[a], *M. canis* wild type strain, and *M. arginini* strain 27389[a] nucleic acids. This testing was run side-by-side on the iiPCR and qPCR assays with 3 replicates for each pathogen.

Absolute quantitation of DNA targets

Digital droplet PCR (ddPCR) was performed on the same set of standard reference nucleic acid as used for the LOD determination for the DNA targets FHV, *Bordetella* species, *C. felis*, and *M. felis*. Primers and probes used for the ddPCR were the same as those used for the qPCR assays. The ddPCR utilized a 25µL reaction comprised of: 12.5µL ddPCR Supermix[i], 5µL of DNA target, the assay appropriate qPCR primer/probe concentrations, and PCR grade H_2O to volume. The generation of droplets and the reading of droplet number were performed per manufacturer instructions[11]. The ddPCR thermocycling conditions were the same as the qPCR assays.

Results from the ddPCR analyzer[i] were recorded for absolute quantification of the number of DNA copies per 5µL DNA target for each dilution. These were then

extrapolated to template volume utilized in the qPCR and iiPCR reactions and reported as such. The ddPCR was not performed for FCV as it is an RNA target and there was difficulty with the RT step and this technology.

Clinical samples and selection methods

A total of 187 oro-pharyngeal clinical swabs collected from cats in shelters were available for this study. Of the 187 swabs collected, 69 were from cats exhibiting signs of FURD and 118 were from asymptomatic cats. For each pathogen target 30 known positive samples and 30 known negative samples were randomly selected (samples that had previously tested positive on the qPCR reference assays). The use of 30 positive and negative samples allowed for 5% error with 95% confidence when the analytical sensitivity and specificity were estimated at 98%. Figure 2.2 demonstrates the flow diagram used to create categories of swabs with various characteristics, from which the 30 positive and negative samples were randomly selected. Samples were divided into those from cats with and without FURD, those previously determined to be negative or positive for the assay specific pathogen, and based on the number of other pathogens detected in the sample. Random selection of samples was performed using a random number generator[k]. All samples with suspect Cts or those whose repeat testing was inconsistent were omitted.

All samples were run side-by-side on the iiPCR and reference assays. Samples were blinded by one author and the iiPCR and qPCR were run by two separate authors who did not know the blinded sample numbers. Results were reviewed after the runs for a specific assay were completed at which time the samples were un-blinded and

replacement samples were chosen when samples behaved as described above. In the case of the *Bordetella* species target four serial dilution replicates of stock *B. bronchiseptica* pathogen were utilized as positive samples as a limited number of positive clinical samples were available. *C. felis* only had one positive clinical sample, which was an ocular sample, so the positive samples were composed of randomly selected and blinded serial dilution replicates of stock pathogen.

Statistical Analysis

Measures of agreement were defined as analytical sensitivity, specificity, and Cohen's kappa values for the purpose of evaluating the iiPCR reagents. Data were entered into an open source online generator[1].

Results

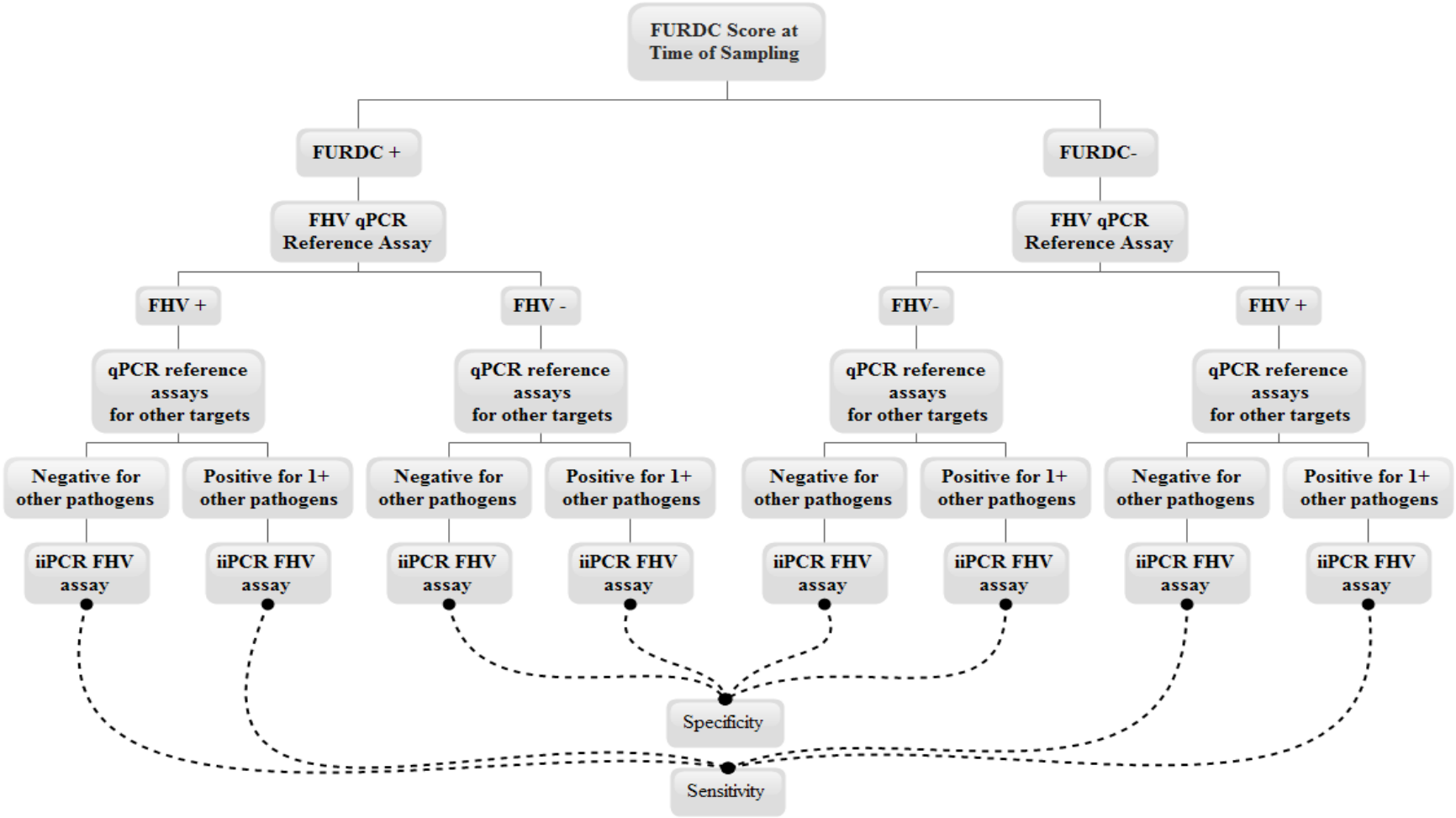
Stage 1: Assay Selection

The first stage of the validation pipeline was completed with selected qPCR reference assays and developed iiPCR point of need reagents detecting the same gene targets for each pathogen(Table 3.1). The percentage of overlap in amplified nucleic acid sequences between the qPCR and iiPCR was 0% for FHV, 91.9% for FCV, 12.8% for *Bordetella* species, 78.6% for *C. felis*, and 73.6% for *M. felis*.

Stage 2: Validation of qPCR Reference Assays

As the second stage of the validation pipeline, three scientific replicates resulting

Figure 2.2. Diagram of the clinical sample selection. This figure diagrams the selection of samples based on characteristics including whether the sample came from an animal with respiratory disease or not, whether the sample contained the target pathogen or not, and whether the sample was positive for other FURD pathogens and how many were present.



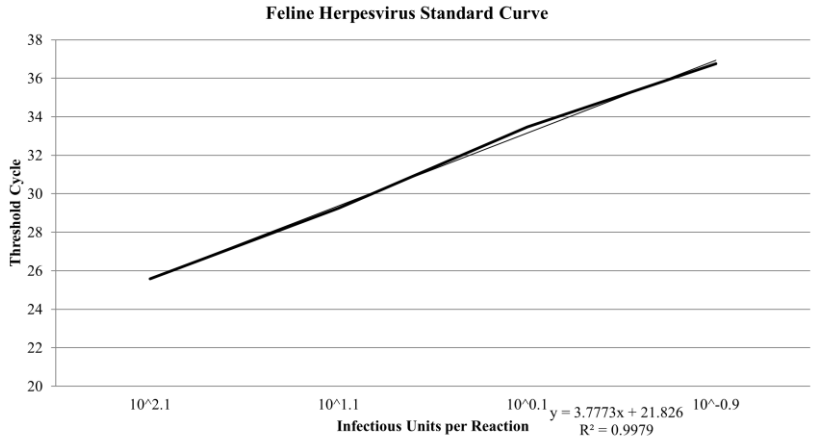
extracted standard nucleic acid. Table 3.2 and Figure 2.3 demonstrate the establishment of a standard curve for FHV through internal laboratory validation of LODs. Similar results were determined for the other target pathogens. The LODs determined for the reference assays were $10^{0.1}$ and $10^{-1.10}$ $TCID_{50}$ for FHV and FCV respectively, $10^{-0.03}$ and $10^{-0.1}$ CFU for *B. bronchiseptica* and *M. felis* respectively, and $10^{-1.4}$ $CEID_{50}$ for *C. felis*.

Table 3.2. Stage 2 reference assay validation demonstrated through establishment of Limits of Detection for Feline Herpesvirus using 9 replicates of stock standard pathogen.

Target	IFU*	Ct	Pos (n=9)
FHV	10^2.1	25.58 ± 0.46	9
	10^1.1	29.26 ± 0.62	9
	10^0.1	**33.48 ± 0.78**	**9**
	10^-0.9	36.76 ± 0.41	5
	10^-1.9	ND	0

*IFU = Infectious units per reaction

Figure 2.3. Feline Herpesvirus Standard Curve on the qPCR Laboratory Reference Assay. This graph demonstrates the standard curve for FHV pure culture control which was utilized to validate the reference laboratory qPCR assay. Standard curve was created via the running of a serial dilution set of the standard DNA in 9 replicates.



Stage 2: 100% Limits of Detection for Point of Need Reagents vs. Reference Assays

The 100% LODs were determined for the iiPCR reagents using 4 replicates run side-by-side with 3 replicates on the qPCR assays of 2 dilutions above and 2 dilutions below the previously determined qPCR LOD. The qPCR assays utilized 2.5µl of DNA in 12.5µl reactions for FHV and *C. felis*, 5µl of DNA/RNA for *B. bronchiseptica* and FCV, and 4µl of DNA for *M. felis*. The iiPCR utilized 5µl of nucleic acid template in 50µl reaction for all targets. The results are displayed in Table 3.3. The LODs were equivalent for FHV and FCV with both assays detected below 1 infectious unit. The qPCR was 1 log more sensitive than the iiPCR for *B. bronchiseptica*, *C. felis*, and *M. felis*. The iiPCR was able to detect $10^{0.97}$ CFU for *B. bronchiseptica*, $10^{-0.3}$ $CEID_{50}$ for *C. felis*, and 10^{1} CFU for *M. felis*.

This shift in LOD shown in table 5 for the bacterial pathogens could be due to the percentage of nucleic acid in the assays, as the iiPCR uses approximately half the percentage of nucleic acid template that the qPCR uses, or could be due to variations in the DNA sequences amplified by the primer sets. To determine if this shift was due to the percentage of DNA in the reaction, the DNA in the iiPCR reactions for *Bordetella* species and *M. felis* was doubled (10µL DNA per 50µL reaction) so that there was equivalent input utilized and the dilution below the determined iiPCR LOD was rerun in triplicate. Normalizing for *M. felis* resolved discrepancy in LOD (100% LOD=$10^{0.3}$), and normalizing for *B. bronchiseptica* only partially resolved the discrepancy in LOD (100% LOD=$10^{0.97}$). The *C. felis* assay was not evaluated at this time due to the sensitivity of the assay and the biological relevance of its level of detection.

Table 3.3. LODs for comparison of the reference laboratory qPCR assays and the point of need iiPCR reagents utilizing serial dilutions of stock pathogens in side-by-side runs. iiPCR was performed using 5µl of nucleic acid in total 50µl reactions while qPCR was performed using 2.5µl per 12.5µl reaction for FHV and *C. felis*, 4µl per 25µl reaction for *M. felis*, and 5µl per 25µl reaction for *B. bronchiseptica* and FCV.

Target	qPCR				iiPCR			
	IFU*	Genome equivalents†	Ct	Pos (n=3)	IFU*	Genome equivalents	Fl ratio‡	Pos (n=4)
FHV	10^2.1	66.67 ± 2.36	24.97 ± 0.11	3	10^2.4	129.33 ± 4.73	4.07 ± 0.15	4
	10^1.1	4.21 ± 0.18	29.54 ± 0.14	3	10^1.4	8.42 ± 0.36	4.41 ± 0.15	4
	10^0.1	**0.27 ± 0.05**	**33.00 ± 0.16**	**3**	**10^.4**	**0.54 ± 0.10**	**3.48 ± 0.63**	**4**
	10^-0.9	0.01 ± 0.02	37.08 ± 0.74	2	10^-0.6	0.03 ± 0.05	3.51 ± 0	1
	10^-1.9	0.01 ± 0.03	ND	0	10^-1.6	0.03 ± 0.05	ND	0
FCV	10^.90	NP	30.68 ± 0.09	3	10^1.2	NP	5.00 ± 0.16	4
	10^-0.10	NP	35.20 ± 0.34	3	10^0.2	NP	4.99 ± 0.19	4
	10^-1.10	NP	**37.20 ± 0.49**	**3**	**10^-0.8**	NP	**4.30 ± 1.08**	**4**
	10^-2.10	NP	41.25 ± 1.29	2	10^-1.8	NP	1.44 ± 0.03	2
	10^-3.10	NP	ND	0	10^-2.8	NP	ND	0
B. bronchiseptica	10^1.97	20.23 ± 1.55	29.50 ± 0.36	3	10^1.97	20.23 ± 1.55	2.38 ± 0.54	4
	10^0.97	4.53 ± 0.84	33.38 ± 0.16	3	**10^0.97**	**4.53 ± 0.84**	**2.48 ± 1.02**	**4**
	10^-0.03	**0.13 ± 0.04**	**37.35 ± 1.33**	**3**	10^-0.03	0.13 ± 0.04	1.47 ± 0.23	2
	10^-1.03	0.03 ± 0.05	ND	0	10^-1.03	0.03 ± 0.05	ND	0
	10^-2.03	ND	ND	0	10^-2.03	ND	ND	0

*IFU: Infectious units per reaction measured in TCID50 for FHV and FCV, CFU for *B. bronchiseptica* and *M. felis*, and CEID50 for *C. felis*.

†Genome equivalents: Detected by absolute ddPCR for all DNA targets; NP = not performed.

‡Fl ratio: Florescence ratio as determined by the internal computer of the POCKIT™ system is the ratio used to determine positive from negative samples, the average ratio and confidence interval were calculated using only positive wells; ND = not detected.

Table 3.3 Continued.

Target	qPCR				iiPCR			
	IFU*	Genome equivalents†	Ct	Pos (n=3)	IFU*	Genome equivalents	Fl ratio‡	Pos (n=4)
C. felis	10^0.4	59.17 ± 9.82	24.92 ± 0.06	3	10^0.7	118.33 ± 19.63	3.58 ± 0.13	4
	10^-0.6	6.185 ± 0.48	29.04 ± 0.10	3	**10^-0.3**	**12.37 ± 0.95**	**3.51 ± 0.27**	**4**
	10^-1.4	**0.685 ± 0.27**	**35.52 ± 0.19**	**3**	10^-1.3	1.37 ± 0.55	1.98 ± 0.81	2
	10^-2.4	0.06 ± 0.05	36.03 ± 0	1	10^-2.3	0.12 ± 0.10	2.49 ± 0	1
	10^-3.4	0.14 ± 0.24	ND	0	10^-3.3	0.28 ± 0.48	ND	0
M. felis	10^1.9	8.91 ± 1.44	27.73 ± 0.03	3	10^2	11.14 ± 1.79	4.48 ± 0.16	4
	10^0.9	1.43 ± 0.17	31.64 ± 0.13	3	**10^1**	**1.79 ± 0.21**	**3.55 ± 0.72**	**4**
	10^-0.1	**0.24 ± 0.16**	**35.11 ± 0.28**	**3**	10^0	0.29 ± 0.20	1.33 ± 0	1
	10^-1.1	0.07 ± 0	ND	0	10^-1	0.08 ± 0	ND	0
	10^-2.1	ND	ND	0	10^-2	ND	ND	0

Stage 2: Exclusivity of Reference and Point of Need Reagents

Exclusivity testing was performed for both iiPCR and qPCR at 10^4 concentration of nucleic acid above 100% reaction LOD for all specific target reagents resulted in no false positives. Table 3.4 demonstrates exclusivity results. Slightly expanded *M. felis* exclusivity was performed. No cross-reactivity was demonstrated for any pathogens.

Table 3.4. Microbial exclusivity panel used for validation of the qPCR laboratory reference assay and the point of need iiPCR reagents for the target pathogens.

	Amplification with the specific qPCR and iiPCR reagents				
Microbe	FHV	FCV	*Bordetella* species	*C. felis*	*M. felis*
FHV	+*	-	-	-	-
FCV	-	+	-	-	-
B. bronchiseptica	-	-	+	-	-
C. felis	-	-	-	+	-
M. felis	-	-	-	-	+
M. bovis	NP	NP	NP	NP	-
M. canis	NP	NP	NP	NP	-
M. arginini	NP	NP	NP	NP	-

*+, positive amplification occurred; -, no amplification occurred
NP=Not performed.

Stage 3: Selection of Clinical Samples

Table 3.5 reports the characteristics that made up the clinical samples tested for each pathogen target. Clinical sample substitutions were made for both *C. felis* and *Bordetella* species due to a lack of positive clinical samples. For *Bordetella* species, 87% of pathogen positive samples were clinical samples while 13% were substituted serial dilution standards. *C. felis* had only 3% pathogen positive clinical samples and instead

utilized 97% substituted serial dilution standards. Overall, samples were broken into 4 categories based on whether they were pathogen positive or negative and randomly selected from those categories to ensure a clinical sample population that reflected both multiple pathogen infections but also samples from cats with inflammation.

Stage 3: Measures of Agreement Utilizing Clinical Sample Sets

For the stage 3 Analytical laboratory measures of agreement testing, 30 positive and negative samples were run for each target pathogen and analytical sensitivity, specificity, and kappa values were calculated. Measures of agreement performed were sensitivity and specificity and these are reported in Table 3.6. The sensitivity and specificity for FHV, FCV, *Bordetella* species, and *M. felis* were all 90% or greater and 93% of greater respectively. The kappa values for these assays were also all 0.9 or greater. *C. felis* had a sensitivity of 80% and a specificity of 100%. The kappa value for *C. felis* was 0.8.

Discussion

This study illustrates the use of the 5 stage pipeline for validation of point of need field deployable devices. Validation of the laboratory reference qPCR assays and the iiPCR point of need reagents for FURD pathogens were used as proof of concept. This study demonstrates the effectiveness of this systematic approach to validation of a new point of need detection system and the development of reagents for those systems. Stage 1 selection of laboratory qPCR reference assay and iiPCR primer and probe oligonucleotides resulted in detection of similar amplicon sequences for the pathogens FCV, *C. felis*, and *M. felis* and detection of different amplicon sequences for FHV and

Table 3.5. Characteristic make-up of the clinical samples tested for each pathogen target including the number of samples that were positive and negative for the target, whether those samples were from cats that were positive or negative for FURD and if the samples tested positive or negative for other target pathogens (with percentages in parenthesis).

Target Reagent	Target positives (n=30)					Target negatives (n=30)			
	FURD +		FURD-		Sub*	FURD+		FURD-	
	0 OP†	1+ OP	0 OP	1+ OP		0 OP	1+ OP	0 OP	1+ OP
FHV	12 (40)	6 (20)	9 (30)	3 (10)	0 (0)	7 (23)	9 (30)	7 (23)	7 (23)
FCV	3 (10)	17 (57)	5 (17)	5 (17)	0 (0)	2 (7)	8 (27)	6 (20)	14 (47)
***Bordetella* sp.**	3 (10)	12 (40)	8 (27)	3 (10)	4 (13)	7 (23)	9 (30)	6 (20)	8 (27)
C. felis	1 (3)	0 (0)	0 (0)	0 (0)	29 (97)	13 (43)	0 (0)	9 (30)	8 (27)
M. felis	6 (20)	9 (30)	6 (20)	9 (30)	0 (0)	5 (17)	11 (37)	7 (23)	7 (23)

* Sub = Serial Dilution substitutes for samples without 30 positive clinical samples available

†OP = Other pathogens present

Table 3.6. Measures of agreement between the qPCR laboratory reference assay and the point of need iiPCR reagent utilizing clinical samples, including the assay results for 30 positive and 30 negative blinded clinical samples, the sensitivity, specificity, and Cohen's Kappa values (confidence intervals in parenthesis).

Target	Platform	Assay Results		Measures of Agreement		
		Positive (n=30)	Negative (n=30)	Sensitivity	Specificity	Kappa
FHV	iiPCR	29	29	96.7 (83.3,99.4)	96.7 (83.3,99.4)	0.93 (0.68-1.19)
FCV	iiPCR	29	28	96.7 (83.3,99.4)	93.3 (78.7, 98.2)	0.90 (0.65-1.15)
Bordetella* species	iiPCR	27	30	90.0 (74.4, 96.5)	100.0 (88.7, 100.0)	0.90 (0.65 - 1.15)
C. felis*	iiPCR	24	30	80.0 (62.7, 90.5)	100.0 (88.7, 100.0)	0.80 (0.55 - 1.05)
M. felis	iiPCR	27	30	90.0 (74.4, 96.5)	100.0 (88.7, 100.0)	0.90 (0.65 - 1.15)

**Bordetella* species and *C. felis* testing utilized substituted serial dilution standards due to a lack of positive clinical samples

Bordetella species. All assays and reagents targeted identical genes for each pathogen.

Stage 2 validation determined the LOD for pure-culture of each pathogen on both systems. Additionally, the LODs of the reference qPCR assay were demonstrated to be consistent and reproducible through 12 total replicates. The qPCR and iiPCR reactions were equivalent in LOD for both the FHV and FCV targets, with FHV detection at 1-2 $TCID_{50}$ or less than 0.5 genome equivalents; and FCV detection at 0.08-0.2 $TCID_{50}$. This detection of FHV is 50 times less than the amount of FHV virus used in viral challenge models[18] to induce infection. Both the qPCR and iiPCR were determined to be equally sensitive for the detection of FHV and FCV. The LOD for M. felis was equivalent when the amount of template nucleic acid was normalized between the qPCR and iiPCR and was then determined to be 0.8-2 CFU and this level of detection also falls below the experimental infectious dose[16].

The qPCR was one log more sensitive in LOD for both *B. bronchiseptica* and *C. felis*. The discrepancy in percentage of nucleic acid template added to reactions lead to possibly a biased result but equalizing the percentage of template did not change the LOD outcome for *B. bronchiseptica* as it did with *M. felis*. This may be due to *B. bronchiseptica* having a large, more complex genome which could be affected by the lack of denaturation stage on the iiPCR or the fact that there was only a 13% overlap in the amplified target sequence between the iiPCR and the qPCR. However this is unlikely to impact the ability of the assay to perform with clinical samples because its only one log shift in sensitivity and biological relevance the LODs for the iiPCR were still more sensitive than the concentration of *B. bronchiseptica* and *C. felis* previously determined

to cause disease through experimental inoculation[4,5]. The iiPCR reactions LODs were overall as sensitive as the qPCR reference assays and useful for the detection of biologically relevant levels of pathogen targets. Limited exclusivity testing and slightly expanded *Mycoplasma* species exclusivity was performed. Exclusivity testing demonstrated that no cross-reactivity was present.

Stage 3 testing determined the analytical measures of agreement for the iiPCR assay in relation to the qPCR reference assays through measures of sensitivity and specificity. The iiPCR was shown to be both sensitive (80% or greater) and specific (93.3% and greater) in relation to the qPCR reference assay for all pathogens. Kappa values for all reagents were above 0.8 and showed a high level of agreement between the qPCR and iiPCR reactions for the clinical samples. The *C. felis* reagent reported the lowest sensitivity (80%) and kappa value (0.8) but this assay also utilized surrogate serial dilutions of pathogen in the place of clinical samples as there was a lack of available positive clinical samples.

Limitations of this study include the limited availability of feline pathogens for exclusivity testing. The *M. felis* qPCR assay had previously been tested for exclusivity against 17 *Mycoplasma* species and the FCV qPCR assay was previously tested for exclusivity against 7 feline pathogens but other assays did not have previous testing reported[1,14]. Further exclusivity testing will be performed before the beginning of stage 4 and field trial validation. Another limitation of this study was the limited positive clinical samples for *C. felis* analytical testing. It should also be noted that this study excluded 'suspect' samples and only tested positive and negative samples with the intent of

obtaining analytical measures of agreement. The exclusion of ‘suspect’ samples in this study inserts spectrum bias, as when tested in the field, suspect samples would be expected to occur.

While this study did have limitations, the experimental design was chosen in order to test each reagent with a wide array of clinical samples composed of both single and multiple pathogens. This was performed in order to test the reagent against clinical samples that best reflect those of the actual feline population. Both the qPCR and iiPCR were required to perform sensitive and specific detection in the face of multiple pathogen samples and samples with increased inflammatory debris and both reactions performed to high standards. Additionally, while there is no true ‘gold standard’ assay used in this study for comparison. The qPCR reference assays utilized went through vigorous internal validation and testing in order to establish an accepted standard of testing within the laboratory for the purpose of comparison and pathogen detection confirmation. This is one of the tenants of the field diagnostic validation pipeline.

All of this testing must be utilized in conjunction with clinical symptomatology and appropriate confirmation from reference qPCR assays, but the ability to quickly determine sick animals pathogen burdens and appropriately isolate and triage diseases with high impacts on their populations, such as FCV is essential. With eight reactions per run, the commercial field-deployable system tested here is designed to fulfill the need of small-scale or satellite facilities. The iiPCR reagents could significantly lower the costs and shorten the sampling-to-result turn-around time from days to a few hours. Furthermore, sensitivity of the iiPCR reagents makes it possible to detect pathogens at

early stages, enabling users to take appropriate bio-security measures in a timely manner. These reagents can also facilitate on-site investigation of disease outbreaks, where relatively low numbers of known diseased animals are required to be tested. The implementation of field deployable devices with the proper checks in place will not only save countless animals lives in shelter populations where disease spread is rapid and often fatal but also increase the overall health of our pet population, many of whom are adopted from these shelter environments.

The 5 stage validation pipeline was implemented in this study to validate the iiPCR point of need reaction system for the detection FURD pathogens in feline samples. This study demonstrated that the iiPCR reagents are sensitive for the detection of biologically relevant levels of target pathogens in pure culture and in clinical sample screening. The iiPCR reactions were tested in a lyophilized format, which ensures stability of the reagents during the shipping and storage stages. Future validation of the iiPCR reagents through the application of stages 4, a field trial and development of standard operating procedures, and 5, the implementation of the iiPCR in shelter environments for detection of pathogens will be performed. This study demonstrated that the point of need validation pipeline can be utilized for the rapid development of point of need assays for significant pathogens and that the stringent testing and multi-step approach of the pipeline results in assays that are both sensitive and specific.

Sources and Manufacturers

a. Bacterial and viral reference strains, American Tissue Culture Collection, Manassas, VA.

b. Courtesy of Dr. Ricardo Rosenbusch, Iowa State University, Ames, IA

c. BBL, Becton, Dickerson and Company, Sparks, MD

d. Mycoplasma plates, California Davis

e. Blood agar plates,

f. Copan ESwab 480C and 481C, Copan Diagnostics Inc, CA

g. DNeasy Blood & Tissue Kit and Quick-Start Protocol, QIAGEN, Valencia, CA

h. Quanta Biosciences, Gaithersburg, MD

i. Bio-Rad Laboratories, Hercules, CA

j. GeneReach, Biotechnology Corporation, Taichung, Taiwan

k. Microsoft Corporation, Redmond, WA

l. OpenEpi, online

m. Integrated DNA technologies, Inc., Coralville, IA

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CHAPTER 5: FELINE UPPER RESPIRATORY DISEASE PATHOGENS IN MIDWESTERN SHELTER CATS: A CROSS-SECTIONAL STUDY

Modified from a paper to be submitted to *Preventive Veterinary Medicine*

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Abstract

Background: Feline Upper Respiratory Disease is a disease complex that spreads rapidly through shelter housing environments and is a leading concern and cause of morbidity and mortality for felines in these environments.

Objective: To assess detection of pathogens in ocular, nasal, and oro-pharyngeal samples from cats in a specific geographical region and evaluate their association with disease.

Design: A cross-sectional study.

Setting: Five animal shelters and a teaching hospital in the Iowa/Nebraska region from 2011 to 2012.

Cats: Cats with and without clinical signs of upper respiratory disease were sampled in shelters. Cats with disease were considered self-selected and cats without clinical signs

were then randomly selected through lottery and sampled. Cats recruited at the teaching hospital were self-selected by owners volunteering for participation.

Measurements: Clinical signs were scored by severity by observers. Time in the shelter, age, and sex were also recorded for cats. Detection for pathogens (Feline Rhinotraceitis Virus (FHV), Feline Calicivirus (FCV), *Chlamydophila felis (C. felis)*, *Bordetella* species, *Mycoplasma felis (M. felis)*, the *Mycoplasma gateae (M.gateae)* cluster, and other *Mycoplasma* species) were performed utilizing Real-time Polymerase Chain Reaction diagnostic assays. Fischer's exact testing, logistic regression, and backwards elimination models were performed.

Results: 71 cats with respiratory disease and 119 cats without disease were enrolled in this study. Prevalence of FHV, FCV, *C. felis*, *Bordetella* species, *M. felis*, *M. gateae* cluster, and other *Mycoplasma* species in the total study population was 49%, 19.5%, 3.2%, 23.7%, 40%, 24.7%, and 15.3% respectively. Multivariate modeling showed that detection of *Bordetella* species (odds ratio (OR) = 3.143), FCV (OR = 2.830), and housing cats in shelters for 2 to 6 months (OR = 0.146) were all significantly related to respiratory disease severity. Age of cats and detection of *C. felis* were confounding factors for relationship between *Bordetella* species and respiratory disease.

Limitations: Intra-observer rating of clinical signs may have also resulted in changes in respiratory disease score. The decay of nucleic acids in samples and use of antibiotics in some of the cats may have limited pathogen detection.

Conclusions: There risk factors associated with respiratory disease in this study were the detection of *Bordetella* species and the detection of FCV while being housed in the shelters for 2 to 6 months was a protective factor.

1. Introduction

Feline Upper Respiratory Disease (FURD) is a complex disease characterized by, but not limited to, conjunctivitis, rhinitis, and tracheitis. Several etiological agents have been previously associated with the disease including Feline Rhinotracheitis Virus (FHV), Feline Calicivirus (FCV), *Chlamydophila felis* (*C. felis*), *Bordatella bronchiseptica (B. bronchiseptica)*, and *Mycoplasma felis* (*M. felis*) (Tan, 1974; Hoover and Kahn, 1975; Gaskell and Povey, 1977; Sparkes et al., 1999; Speakman et al., 1999).

FURD occurs commonly in group housed felines such as animal shelters (Dinnage et al., 2003). The level of confinement required in shelters and number of animals present can cause immunological stress. This can lead to high levels of morbidity and mortality associated with FURD (Dinnage et al., 2009). FURD was one of the top three diseases of concern identified by western US shelters in 2010 (Steneroden et al., 2011) and was listed as one of the leading medical causes of feline death or euthanasia in shelters (Dinnage et al., 2009). There have been several studies in the past evaluating the association between FURD and various risk factors including pathogenic agents, age, breed, sex, and others (Bannasch and Foley, 2005; Helps et al., 2005; Hartmann et al., 2010; Holst et al., 2010; Burns et al., 2011; Gourkow et al., 2013).

Bannasch and Foley, 2005, evaluated 574 cats in California shelters and found that cats housed near dogs, cats in the shelter for less than 6 days and cats less than 12 months of age were all at a higher risk of developing FURD. Helps et al., 2005, studied FURD in European catteries and found that the pathogens FHV, FCV, and *B. bronchiseptica* were all significantly associated with disease ad that the presence of dogs

and poor hygiene were risk factors. Hartmann et al., 2010, included 41 cats in Germany in an ocular study of FURD which found that there was a higher prevalence of *Mycoplasma* species detected than previously reported. Holst et al, 2010, utilized a case-control methodology to sample the oro-pharynx and conjunctiva of 80 cats in Sweden and found that *M. felis* was associated with clinical signs of FURD. Burns et al., 2011 evaluated close to 100 cats in a California shelter cats to determine that FHV was present in the majority of multiple pathogen infection. Gourkow et al., 2013, assessed 250 cats in a Canadian shelter and found that cats with FHV and *B. bronchiseptica* had the highest risk of developing FURD.

None of these studies included samples from both the ocular, nasal and oral anatomical sites or took place in the upper Midwest. While studies utilizing Polymerase Chain Reactions (PCR) may have had similar sensitivities and specificities, studies utilizing culture and isolation methods would have markedly different sensitivity and specificities of the testing modalities. PCR is also more sensitive than culture methods for many of the FURD pathogens. Additionally, the studies either were case-control and included even number of healthy and diseased cats or only sampled from cats with FURD, making it difficult to assess the pathogen burden in the general shelter population.

The objective of this study was to assess detection of pathogens in ocular, nasal, and oro-pharyngeal samples from cats in a specific geographical region and evaluate their association with disease using sensitive uniform methodology for pathogen detection and a large number of clinically normal cats.

2. Material and Methods

2.1. Study Design

This study utilized a cross-sectional design with populations of felines housed in animal shelters in the Iowa/Nebraska region of the Midwest and client-owned cats from the Lloyd Veterinary Medical Center (LVMC) at Iowa State University College of Veterinary Medicine. Felines in participating shelters were scored for clinical signs of FURD and sampled once. Felines seen at LVMC, whose owners consented to their participation, received the same scoring and sampling. Table 1 reports the demographics of the cats sampled. Conjunctival, oro-pharyngeal, and nasal swabs were collected from each participant. Sampling occurred from June 2011 to August 2012. Samples were tested for the presence pathogens (FHV, FCV, *C. felis, Bordetella species, M. felis*, *M. gateae* cluster, and other *Mycoplasma species*) via Real-Time Polymerase Chain Reactions (qPCR). Comparison of clinical FURD score, pathogen presence, time in the shelter, age, and sex were then statistically analyzed.

2.2. Shelters and Clinic

This cross-sectional study recruited the participation of animal shelters in the Iowa/Nebraska region and clients from the LVMC from June 2011 to July 2012. An enrolled shelter was visited several times during the study time period and at visit cats were enrolled in the study, scored, and sampled. Sampled occurred on the date of enrollment and no follow-up sampling was performed. Five animal shelters participated in this study and are referred to as A, B, C, D, and E. Shelters A and B were located in rural communities while shelters C, D, and E were located in urban communities. All

shelters were involved with animal control in either their city or county. All shelters housed both canines, felines, and assorted other domestic animal species. All shelters held stray cats for a minimum of 7 days before cats became shelter property.

The Lloyd Veterinary Medical Center is located at Iowa State University College of Veterinary Medicine, in Ames, IA and provides clinical services to mostly client-owned companion animals, exotics, and large animal species. If clients gave consent, cats were sampled while at appointment. Reasons for appointments included illness and preventative medicine.

2.3. Feline participants

Felines considered property of the participating shelters during the study time period were eligible to participate in the study and were only sampled once. Cats under the age of 8 weeks or less than 2 pounds in weight were excluded from this study. Shelter personal were asked to identify cats exhibiting clinical signs and symptoms of FURD in either their isolation or general populations. Cats that were safe to handle and exhibiting signs were considered self-selected for inclusion in the study. An effort was made to sample one cat without clinical signs for every cat with clinical signs. Healthy cats, cats without clinical signs of FURD, were randomly selected from the general population utilizing the Microsoft Excel Random Number Generator. Following best practices for disease prevention and biosecurity, all healthy animals and animals maintained in the general adoption population were sampled prior to those in isolation. In the shelters, isolation was considered any room that staff used to separate animals with clinical disease from the general adoption population. Felines presenting to the LVMC were

considered eligible to participate if owner consent was obtained at which point the feline was scored and sampled.

The protocol for feline use was approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University.

2.4. Measures for outcomes and variables

The outcomes of interest were FURD scores. FURD was determined using a clinical score card described below and the levels included: no clinical disease (0), mild disease (1), and severe disease (2). The variables of interest were the presence or absence of FHV, FCV, *C. felis*,

Table 4.1. Demographic characteristics of the 190 sampled cats included in this study.

Variable	Classes	Fraction	Frequency (%)
Shelter	A	24/190	12.6
	B	46/190	24.2
	C	14/190	7.4
	D	47/190	24.7
	E	45/190	23.7
	LVMC	14/190	7.4
Room in shelter	Adoption	88/176	50
	Isolation	68/176	38.6
	Queening	4/176	2.3
	Other	16/176	9.1
Time in shelter (days)	≤ 30	78/141	55.3
	>30 and ≤60	33/141	23.4
	>60 and ≤180	28/141	19.9
	>180	2/141	1.4
Sex	Male	98/189	51.6
	Female	91/189	47.9
Age (months)	≤ 2	9/190	4.7
	>2 and ≤6	53/190	27.9
	>6 and ≤12	17/190	8.9
	>12 and ≤24	16/190	8.4
	>24	95/190	50
Source	Stray	16/43	37.2
	Owner surrender	27/43	62.8

Bordetella species, *M. felis*, *M. gateae* cluster, and other *Mycoplasma* species. Other variables and potential confounders included were: time spent in the shelter, age at time of sampling, and sex. Time in was measured in days. Age was measured in months. Sex was defined as male or female, regardless of whether the animal was intact or surgically altered.

2.5. Data sources/measurements

FURD scoring and the assays utilized for pathogen detection required measurements to be taken. All other variables were sourced from shelter records when available. Age was an estimate and was either reported on surrender or determined through dental exam.

2.5.1. FURD Score Card and Scale

At sampling cats were evaluated using a clinical score card by either a trained veterinarian or veterinary student. The overall general demeanor of the cat was noted as normal, depressed or severely depressed. Evaluators also noted the name/number of the cat and any distinct markings to avoid repeat sampling. Score cards also provided areas for other notes and comments on the cats such as any treatment being administered or clinical signs not related to upper respiratory disease but related to the general wellness of the cat. Characteristics evaluated were modeled from a previous study with modification (Bannasch and Foley, 2005). Conjunctivitis and keratitis were noted as either present or absent. Ocular and nasal discharge were marked as either normal (absent), clear (serous), or muco-purulent. Sneezing was normal (absent), occasional, or frequent/severe. Respiratory effort was evaluated as no effort, moderate effort, or dyspnea. Gingivitis,

plaque, and stomatitis/faucitis were evaluated on a scale of 0-3, 0 being normal (absent), 1 being mild, 2 being moderate, and 3 being severe.

A completed score card was used by the primary investigator to complete a total FURD score. This was assigned utilizing the "case definition and severity score" from a previous study with one modification (Bannasch and Foley, 2005). Based on this system, no clinical disease was noted if only mild serious ocular or nasal discharge or mild signs in one area were noted. Mild clinical disease was defined as score of 1 in several sites or the presence of purulent discharge in one site and mild serious ocular or nasal discharge with other clinical signs. Severe disease was all scores higher, including purulent discharge from the eyes and nose, severe signs in one location, and persistent sneezing during the examination. While the score cards were filled out by several individuals, the assignment of severity score was performed by a single individual to ensure consistency.

2.5.2. Sample collection and qPCR for pathogen detection

Samples in shelters were collected from each cat identified either as a FURD case or the randomly selected control. Individual conjunctival swabs were taken from both the right and left eyes when possible (Copan ESwab 481C, Copan Diagnostics Inc, CA). If nasal discharge was present, a third dry sterile nylon flocculated swab was rolled along the nasal planum. Finally, a deep oropharyngeal swab was taken (Copan ESwab 480C, Copan Diagnostics Inc, CA). All samples were kept cool and transported to the laboratory for processing as soon as possible. Samples collected in the teaching hospital were collected using the same material and methods but were then stored in refrigeration and were collected daily to be processed in the laboratory. Once in the laboratory all

samples were checked for proper labeling and then stored at -80^{o}C. Nucleic Acid extraction was performed as described in a previous study (Donnett et al.,Chapter 4).

2.5.3. qPCR assays for FHV, FCV, *C. felis*, *Bordetella* species, and *M. felis*

The qPCR was performed for FHV, FCV, *C. felis*, *Bordetella* species, and *M. felis* using primers, probes, and thermocycling procedures previously described (Donnett et al., Chapter 4). For each target pathogen, a standard curve was created and the analytical sensitivity was determined. Samples were run in duplicate wells. The PCR positive control was specific to each assay were aliquots of the same, consistent, pure culture dilution of pathogen. Ct was standardized to the positive controls, thus normalizing fluorescence between separate runs (Thesis Chapter 4 Table 3.1)

The standard curve for FHV demonstrated the assay to be sensitive up to 0.125 $TCID_{50}$ per reaction with an R^2 of 0.998 and an amplification efficiency of 85.95%. The standard curve for FCV demonstrated the assay to be sensitive up to 0.07925 $TCID_{50}$ per reaction with an R^2 of 0.9718 and an amplification efficiency of 90.56%. The standard curve for *C. felis* demonstrated the assay to be sensitive up to 0.0025 CEID50/reaction with an $R^2 = 0.9976$ and an amplification efficiency of 90.42%. The standard curve for *Bordetella* species demonstrated the assay to be sensitive up to the 9.4 CFU per reaction with an R2 of 0.9988 and an amplification efficiency of 87.60% . The standard curve for *M. felis* demonstrated the assay to be sensitive up to 1.2 CFU/reaction with an $R^2 =$ 0.9969 and an amplification efficiency of 107.71%. Amplification efficiency was performed using an online calculator (Agilent Genomics, http://www.genomics.agilent.com/bioCalcs.jsp).

2.5.4. Pan-Myco SYBR qPCR for *M. gateae* cluster and other *Mycoplasma* species

The *M. gateae* cluster and other Mycoplasma species were detected through the use of the Pan-Myco SYBR qPCR assay as previously described (Donnett et al., Chapter 3). The standard curve for the *M. gateae* cluster demonstrated the assay to be sensitive up to 3 CFU per reaction with an R^2 of 0.9957 and an amplification efficiency of 78.60%. This assay uses a single set of primers that bind to a conserved region of the *Mycoplasma* genome thus allowing for the amplification of any *Mycoplasma* species present in a sample (Donnett et al., Chapter 3). As such, the assay was tested for analytical sensitivity and specificity for representative *Mycoplasma* species.

2.6. Bias

While all personnel involved in sampling during this study were IACUC approved and instructed on sampling methods, throughout the course of the study there were three people involved in evaluation and scoring of animals for clinical signs and sample collection. This change in personnel could lead to classification bias in clinical signs thus affecting the outcome of the FURD score and detection bias if sampling methods were not identical across the samplers. There is also the possibility of selection bias as the cats selected as cases were identified by shelter personnel and may not have been exhibiting clinical signs at the time of sampling. This selection bias led to the uneven distribution of clinically ill and healthy cats (86 and 104 cats respectively).

2.7. Sample size

The number of cats with FURD during the time period of this study in these shelters and their asymptomatic counterparts determined the number of cats in this study. Additionally, any cats from the LVMC that received client-consent were included.

2.8. Quantitative variables

For analysis, the variable 'age' was broken up into 5 categories. These categories were ≤ 2, >2 and ≤6, >6 and ≤12, >12 and ≤24, and >24 months. These categories were chosen for age breakdowns because they give an adequate assessment of the different stages of growth for a kitten and include age ranges that can be determined using dental scoring. Once a cat is older than 2 years and considered an adult it becomes difficult to determine its age, if for example it is a stray and has no past known history. For most of the respiratory pathogens, cats carry maternal immunity till around 2 months of age.

The variable 'time in' days in the shelter was broken up into 4 categories. These categories were ≤ 30, >30 and ≤60, >60 and ≤180, and >180 days in the shelter. This break down was selected because it allowed assessment based roughly on the number of months spent in the shelter.

2.9. Statistical Methods

Statistical analysis was performed using the statistical software package SAS (SAS Institute Inc., Cary, NC). First descriptive analysis was performed for all of the variables for all of the FURD score levels (0,1,2). After descriptive analysis was performed, Fischer's exact testing was performed. Fischer's exact testing was used to evaluate the differences between models with the outcomes "FURD score 0,1,2", "FURD

score 0-1, 2", and "FURD score 0, 1-2". It was determined that the outcomes 1 (mild disease) and 2 (severe disease) could be combined, and further modeling was performed using the outcome "FURD score 0, 1-2".

Modeling was first performed using the outcomes any pathogen detection and multiple pathogen detection. Any pathogen detection was a dichotomous variable while multiple pathogen detection was treated as a categorical variable with the classes reflecting the number of pathogens detected overall in a cat, ie. 0-1, 2,3,4,5. First univariate modeling, maximum likelihood point estimate analysis of each variable, and the odds ratio and 95% confidence interval were determined for these variables. Than an multivariate model adjusting for age, time in, and sex was analyzed.

Next univariate models were run for all the individual variables. The variables Sex, FHV, FCV, *C. felis*, *Bordetella* sp., *M. felis*, *M. gateae* cluster, and other *Mycoplasma* were all analyzed through logistic regression as dichotomous variables. The variables Age and Time in shelter were analyzed through logistic regression as categorical variables as described above. Each variable was first analyzed using a univariate linear logistic regression model with "FURD score 0, 1-2" as the outcome and the variable in question as the explanatory variable for using a maximum likelihood method of estimation. As a single sample was taken from each subject an adjusted multivariate model was deemed to be appropriate.

Following univariate modeling, maximum likelihood point estimate analysis of each variable, and determination of the odds ratio and 95% confidence interval, an adjusted multivariate model including all variables as the explanatory variables and

"FURD Score 0,1-2" as the outcome was analyzed. In the multivariate model Sex, FHV, FCV, *C. felis*, *Bordetella* sp., *M. felis*, *M. gateae* cluster, and other *Mycoplasma* were included as dichotomous variables and Age and Time in shelter were included as grouped categorical variables. All cats with missing data values were excluded from multivariate analysis and the working feline sample number for this modeling was 141 cats.

The initial model for logistic regression formulated above had "FURD score 0, 1-2" as the outcome variable and included all possible main effects (sex, age, time in the shelter, FHV, FCV, *C. felis*, *Bordetella* sp., *M. felis*, *M. gateae* cluster, and other *Mycoplasma*). The model was then simplified using hierarchical backwards elimination. This procedure was run and the result was considered the unadjusted final model. Predictors with the largest p-value above 0.05 were removed from the model one at a time and assessed for confounding. A variable was considered to be a confounder if its inclusion in the multivariate model altered the OR for the association between "FURD score 0, 1-2" and *Bordetella* sp. by 10% or more. If the variable was found to be a confounder it was retained in the model. Backwards elimination was continued until all variables in the model had either a significant p-value of equal to or less than 0.05, or were found to be confounders. This was determined to be the final adjusted model. This final adjusted model was then used to find the maximum likelihood point estimate analysis for each variable and the odds ratio and 95% confidence intervals were also determined. The p-value was considered significant at <0.05.

3. Results

3.1. Feline demographics

5 shelters and 14 clients at the LVMC consented to participation in this study. A total of 190 cats were samples for this study including: 24 (12.6%) from shelter A, 46 (24.2%) from shelter B, 14 (7.4%) from shelter C, 47 (24.7%) from shelter D, 45 (23.7%) from shelter E, and 14 (7.4%) from the LVMC. The wide range in numbers of cats sampled at shelters was dependent upon the cat population in the shelters and the number of times the shelter was visited for sampling. Shelters farther than 60 minutes from Ames, IA were sampled at a single visit. The demographic breakdown of feline participants included in this study is displayed in **Table 1**. Of the 190 cats, 176 were members of shelter populations. Of these 176 cats, 141 had data available and collected on their length of time spent in the shelter and 43 had data available and collected on their source prior to shelter housing. One shelter cat had missing data related to sex. The majority of shelter cats in this study were housed in the general adoption population (50%) with the isolation housing being the second largest population (38.6%). The large majority of shelter cats sampled were also owner surrenders (62.8%). More than half of the shelter cats (55.3%) had been in the shelter for 30 days or less. The overall cat population was equally distributed between male (51.6%) and female (47.9%). The majority of cats were either between the age of 2 and 6 months (27.9%) or older than 24 months (50%).

All 190 participating cats were clinically scored for FURD. Table 4.2 demonstrates the number of cats with specific clinical signs and their assigned overall FURD severity score. The most prevalent clinical signs observed in cats were serous

ocular discharge (24.2%), sneezing (25.8%), gingivitis (21.1%), and a body condition score of less than 3 on a scale of 1-5 (16.3%). While all these clinical signs can be caused by factors other than FURD, the combination of multiple of these in an individual was considered clinical signs of disease. Cats which appeared most severely affected by upper respiratory disease had higher prevalence of serous ocular discharge (11.1%), sneezing (17.4%), and stomatitis (5.3%) than cats with no or mild disease scores. The majority of cats with low body condition scores did not have clinical signs of disease (11.6%).

Table 4.2. The number (and percentage) of cats with specific clinical signs and the overall assigned FURD severity score.

Clinical signs	FURD Severity Score			Total
	0 (none)	1 (mild)	2 (severe)	
Conjunctivitis	2 (1.1)	7 (3.7)	9 (4.7)	18 (9.5)
Keratitis	0 (0)	2 (1.1)	3 (1.6)	5 (2.6)
Serous ocular discharge	13 (6.8)	12 (6.3)	21 (11.1)	46 (24.2)
Purulent ocular discharge	0 (0)	2 (1.1)	6 (3.2)	8 (4.2)
Serous nasal discharge	2 (1.1)	5 (2.6)	12 (6.3)	19 (10)
Purulent nasal discharge	0 (0)	4 (2.1)	11 (5.8)	15 (7.9)
Sneezing	7 (3.7)	9 (4.7)	33 (17.4)	49 (25.8)
Gingivitis	12 (6.3)	15 (7.9)	13 (6.8)	40 (21.1)
Stomatitis/Faucitis	0 (0)	3 (1.6)	10 (5.3)	13 (6.8)
Body condition score < 3	22 (11.6)	3 (1.6)	6 (3.2)	31 (16.3)
Total cats	119 (62.6)	32 (16.8)	39 (20.5)	190

3.2. Total FURD score and pathogen detection

Of the 190 cats sampled in this study, 119 (62.6%) had a clinical FURD score of 0 or no disease. Cats with disease broke down into 32 (16.8%) cats with a FURD score of 1, or mild, and 39 cats with a FURD score of 2 (20.5%), or severe. Table 4.3 provides a breakdown of FURD score and pathogen detection per housing location. With the exception of Shelter C, all shelters had animals sampled that received scores of 0, 1, and

2 for FURD. The majority of the cats sampled in shelters, 111 (63.1%) had no clinical disease.

Table 4.3. Total numbers of cats with each FURD severity score per housing location as well as the prevalence (percent) of detected pathogens in the 5 animal shelters and the LVMC cats.

		Housing location						All
		LVMC	A	B	C	D	E	shelters
Cats sampled		14	24	46	14	47	45	176
FURD Severity Score	0 (none)	8 (57.1)	19 (79.2)	26 (56.5)	11 (78.5)	32 (68.1)	23 (51.1)	111 (63.1)
	1 (mild)	2 (14.3)	3 (12.5)	7 (15.2)	3 (21.4)	5 (10.6)	12 (26.7)	30 (17.1)
	2 (severe)	4 (28.6)	2 (8.3)	13 (28.3)	0 (0)	10 (21.3)	10 (22.2)	35 (19.9)
Pathogens detected	FHV	2 (14.3)	8 (33)	33 (71.7)	1 (7.2)	34 (72.3)	15 (33.3)	91 (51.7)
	FCV	1 (7.1)	3 (12.5)	12 (26.1)	1 (7.2)	16 (34)	4 (8.9)	38 (21.6)
	C.felis	4 (28.6)	0 (0)	1 (2.2)	0 (0)	1 (2.1)	0 (0)	2 (1.1)
	Bordetella sp.	1 (7.1)	3 (12.5)	23 (50)	1 (7.2)	0 (0)	17 (37.8)	44 (25)
	M. felis	4 (28.6)	8 (33)	16 (35)	6 (42.9)	16 (34)	26 (57.8)	72 (40.9)
	M. gateae cluster	8 (57.1)	7 (29)	11 (22.4)	5 (35.7)	11 (23.4)	5 (11.1)	39 (22.2)
	Other *Mycoplasma* sp.	7 (50)	3 (12.5)	8 (17.4)	1 (7.2)	6 (12.8)	4 (8.9)	22 (12.5)

FHV was detected in 91 (51.7%) of the shelter cats with shelters B and D having the highest prevalence of FHV detection at 71-72%. FCV was detected in 38 (21.6%) of the cats with the highest prevalence of detection in shelter D (34%). *C. felis* was only detected in 2 (1.1%) shelter cats, 1 cat from shelter B and one cat from shelter D. The majority of the *C. felis* detection occurred in home-owned cats at the LVMC (28.6%).

Bordetella species were detected in 44 (25%) of the shelter cats with the highest prevalence being in shelter B (50%). Only 1 (7.1%) home-owned cat had *Bordetella* species detected. *M. felis* had the second highest prevalence of the pathogens detected, being found in 72 cats (40.9%). The majority of these detections (36.1%) occurred in cats from shelter E.

M. gateae cluster was detected in 39 (22.2%) of cats in shelters and other *Mycoplasma* species were detected in 22 (12.5%) of cats in the shelters. The other *Mycoplasma* species detected included *M. canis*, *M. hyorhinis*, *M. alkalescens*, *M. cynos*, *M. faucium*, *M. dispar*, *M. buccale*, *M. spumans*, *M. hominis*, *M. bovis*, *M. bovoculi*, *M. maculosum/leopharyngis* cluster, and 2 new unknown species and more information can be found in the previous publication detailing detection and differentiation of these species (Donnett et al., Chapter 3).

The results for the modeling of FURD score severity against pathogen detection can be found in Table 4.4. In the unadjusted odds rate model which includes the data from 190 cats, the odds of having mild to severe FURD if any pathogen was detected was 4.34 (0.98, 19.29) times that of cats with no pathogens detected. This odds ratio has a wide confidence interval and includes the null and the p value (0.054) is just above the significant level of 0.05 but is significant at a level of 0.10. Overall, this provides weak evidence that the detection of any pathogen increases the odds of FURD. Modeling adjusting for age, time in the shelter, and sex of the cats resulted in 3.99 (0.869, 18.336) odds of having mild to severe FURD if any pathogen was detected. Again the p value of

0.0752 is only significant at the 0.1 level and the null is included in the 95% confidence interval.

In the unadjusted odds rate model the odds of having mild to severe FURD if multiple pathogens are detected was only significant for the detection of 4 pathogens in a single cat. The odds of having mild to severe FURD if 4 pathogens were detected was 6.44 (1.524, 27.246) times that of cats with 0 or 1 pathogen detected. The p value for the detection of 4 pathogens in a single cat was 0.0299, although the confidence interval for 4 pathogens detected was very wide, which suggests that this is imprecise. When the model was adjusted for the variables age, time in the shelter, and sex of the cats, no level of multiple pathogen detection was found to be significantly related to FURD.

Table 4.4. Relative rates of any pathogen and multiple pathogens detection and the measures of association with any pathogen detected and multiple pathogens detected.

		Unadjusted Odds Rate			Adjusted Odds Rate		
		Odds ratio	95% CI	P	Odds ratio	95% CI	P
Any pathogen	0	Ref	-	-	Ref	-	-
	1+	4.34	(0.98, 19.29)	0.054	3.99	(0.87, 18.34)	0.0752
Multiple pathogens detected	0-1	Ref	-	-	Ref	-	-
	2	2.53	(1.27, 5.03)	0.2635	3.340	(1.42, 7.88)	0.9186
	3	1.38	(0.54, 3.55)	0.5947	9.469	(0.84, 9.47)	0.9216
	4	6.44	(1.52, 27.25)	0.0299	3.812	(0.71, 20.57)	0.9162
	5	0.69	(0.07, 6.53)	0.3131	-0.001	(<0.001, >999.9)	0.9241

Adjusted odds rate for age, time in the shelter, and sex of the cats

3.3 Univariate and multivariate modeling for FURD scores and all factors

The results from the univariate modeling for all possible main effects are displayed in Table 4.5. Age greater than 24 months, >60 days in the shelter to ≤180 days in the shelter , and *Bordetella* species detection were all associated with the outcome FURD score of mild or severe at the 0.05 significance level. None of these variables had confidence intervals that included the null. The odds of having mild or severe FURD for a cat over 24 months of age was 0.126 (0.025, 0.642) times that of a cat less than or equal to 2 months of age and had a p value of 0.0133. The odds of having mild or severe FURD for a cat in a shelter for greater than 60 days and less than or equal to 180 days was 0.281 (0.097, 0.817) times that of a cat in the shelter for less than or equal to 30 days and had a p value of 0.0415. The odds of having mild or severe FURD for a cat with *Bordetella* species detected was 2.113 (1.071, 4.167) times that of a cat without *Bordetella* species and had a p value of 0.0309. FCV was also associated with the outcome FURD score of mild or severe at the 0.1 significance level. The odds of having mild or severe FURD for a cat with FCV detected was 2.050 (0.992, 4.239) times that of a cat without FCV and had a p value of 0.0527. Overall, when modelled independently, cats older than 24 months of age and cats in the shelter for 3 to 6 months had decreased risk of mild or severe FURD while cats with *Bordetella* species detected and cats with FCV detected were at increased risk of mild or severe FURD.

The results of the multivariate backwards elimination modeling are reported in Table 4.6. The variables *C.felis*, other *Mycoplasma* species, *M. felis*, Age, FHV, sex, and *M. gateae* cluster were removed from the model via backwards elimination in that order.

Once removed, assessment for confounding found that the variables *C. felis* and age significantly increased the OR for *Bordetella* species by more than 10%. Therefore they were replaced in the model and were considered confounding factors. The variables *Bordetella* species, FCV, and time in the shelter all had statistical significance and so remained in the model. The final unadjusted model included the exposure variable of interest *Bordetella* species and the modifying factors FCV and time in the shelter. There were no confounding factors in the unadjusted model. The final adjusted model included the exposure variable of interest *Bordetella* species and the modifying factors FCV, time in the shelter, age, and *C. felis*. This model included the confounding factors age and *C. felis*.

In the unadjusted model, the odds of having mild or severe FURD for a cat with *Bordetella* species detected was 2.529 (1.01, 6.35) times that of a cat without *Bordetella* species and had a p value of 0.0481. The odds for a cat with FCV detected was 3.380 (1.30, 8.83) times that of a cat without FCV and had a p value of 0.0129. The odds for a cat in a shelter for greater than 60 days and less than or equal to 180 days was 0.165 (0.05, 0.54) times that of a cat in the shelter for less than or equal to 30 days and had a p value of 0.0367. In the adjusted model, the odds of having mild or severe FURD for a cat with *Bordetella* species detected was 3.143 (1.19, 8.32) times that of a cat without *Bordetella* species and had a p value of 0.0211. The odds for a cat with FCV detected was 2.830 (1.07, 7.49) times that of a cat without FCV and had a p value of 0.0363. The odds for a cat in a shelter for greater than 60 days and less than or equal to 180 days was.

Table 4.5. Descriptive analysis and univariate point estimates, p-values, and odds ratios for FURD prevalence with risk factors.

Variable	Category	FURD Prevalence 0	FURD Prevalence 1-2	Odds Ratio	95% Confidence Interval	P value	
Age (months)	≤ 2	2 (1.1)	7 (3.7)	Referent	-	-	
	>2 and ≤6	32 (16.8)	21 (11.1)	0.188	(0.35, 0.991)	0.3358	
	>6 and ≤12	10 (5.3)	7 (3.7)	0.20	(0.032, 1.265)	0.5931	
	>12 and ≤24	9 (4.7)	7 (3.7)	0.222	(0.035, 1.422)	0.7705	
	>24	66 (34.7)	29 (15.3)	0.126	(0.025, 0.642)	0.0133	**
Sex	Male	57 (30.2)	41 (21.7)	Referent	-	-	
	Female	61 (32.3)	30 (15.9)	0.684	(0.378, 1.238)	0.2092	
Time In Shelter (days)	≤ 30	44 (31.2)	34 (24.1)	Referent	-	-	
	>30 and ≤60	17 (12.1)	16 (11.3)	1.218	(0.538, 2.755)	0.3841	
	>60 and ≤180	23 (16.3)	5 (3.5)	0.281	(0.097, 0.817)	0.0415	**
	>180	1 (0.7)	1 (0.7)	1.294	(0.078, 21.446)	0.6673	
FHV	Negative	65 (34.2)	32 (16.8)	Referent	-	-	
	Positive	54 (28.4)	39 (20.5)	1.467	(0.813, 2.648)	0.2034	
FCV	Negative	101 (53.2)	52 (27.4)	Referent	-	-	
	Positive	1 (9.5)	19 (10.0)	2.050	(0.992, 4.239)	0.0527	*
C. felis	Negative	115 (60.5)	69 (36.3)	Referent	-	-	
	Positive	4 (2.1)	2 (1.1)	0.833	(0.149, 4.670)	0.8357	
Bordetella sp.	Negative	97 (51.1)	48 (25.3)	Referent	-	-	
	Positive	22 (11.6)	23 (12.1)	2.113	(1.071, 4.167)	0.0309	**
M. felis	Negative	72 (37.9)	42 (22.1)	Referent	-	-	
	Positive	47 (24.7)	29 (15.3)	1.058	(0.581, 1.926)	0.8541	
M. gateae cluster	Negative	90 (47.4)	53 (27.9)	Referent	-	-	
	Positive	29 (15.3)	18 (9.5)	1.054	(0.535, 2.079)	0.8789	
Other Mycoplasma sp.	Negative	100 (52.6)	61 (32.1)	Referent	-	-	
	Positive	19 (10.0)	10 (5.3)	0.863	(0.376,1.977)	0.7273	

Significance levels reported: *=0.1 significance; **=0.05 significance; ***=0.005 significance

Table 4.6. Multivariate point estimates, p-values, and odds ratios for *Bordetella* sp. related to FURD prevalence with both the unadjusted odds rate and adjusted odds rate for confounding variables.

Variables	Category	FURD Score		Unadjusted Odds Rate			Adjusted Odds Rate		
		0	1-2	Point estimate	95% CI	P	Point estimate	95% CI	P
Bordetella sp.	Negative	97 (51.1)	48 (25.3)	Referent	-	-	Referent	-	-
	Positive	22 (11.6)	23 (12.1)	2.529	(1.01, 6.35)	0.0481	3.143	(1.19, 8.32)	0.0211
FCV	Negative	101 (53.2)	52 (27.4)	Referent	-	-	Referent	-	-
	Positive	1 (9.5)	19 (10.0)	3.380	(1.30, 8.83)	0.0129	2.830	(1.07, 7.49)	0.0363
Time in shelter	≤ 30	44 (31.2)	34 (24.1)	Referent	-	-	Referent	-	-
	>30 and ≤60	17 (12.1)	16 (11.3)	0.919	(0.38, 2.25)	0.3158	0.780	(0.31, 1.97)	0.4903
	>60 and ≤180	23 (16.3)	5 (3.5)	0.165	(0.05, 0.54)	0.0367	0.146	(0.04, 0.50)	0.0258
	>180	1 (0.7)	1 (0.7)	0.593	(0.02, 15.35)	0.9485	0.761	(0.03, 20.46)	0.7865

Adjusted odds rate for confounding variable age and *C. felis*.

0.146 (0.04, 0.50) times that of a cat in the shelter for less than or equal to 30 days and had a p value of 0.0258.

4.0 Discussion

This study was an evaluation of FURD in shelter cats and its potential risk factors in a unique geographical location. The sample population was composed of 190 cats from 5 participating shelters and one hospital. Of these cats, 119 (62.6%) had no clinical, 32 (16.8%) cats had a mild FURD, and 39 (20.5%) cats with severe FURD. Cats ranged in age from 2 months to 15 years of age. The study population was equally divided between male and female cats and ranged in time housed in the shelter from 0 days to 285 days. The majority of cats included in the study were owner surrenders (62.8%). FHV was the pathogen most commonly detected in the sampled cats at 51.7% with *M. felis* being the second most common pathogen (40.9%) and *Bordetella* species, FCV, and *M. gateae* cluster (21-25%) all being the third most commonly detected.

Individually significant variables included age, time in shelter, FCV, and *Bordetella* sp. The odds of FURD in cats greater than 24 months of age was 0.126 (0.025, 0.642) with a p value of 0.0133 suggesting older cats are at a decreased odds of FURD as compared to cats 2 months of age or younger. Cats in the shelter for 2-6 months also had significantly decreased odds of FURD. Cats with *Bordetella* species detected and cats with FCV detected had increased odds of FURD.

Multivariate modeling utilized FURD as the outcome of interest and suggested that the detection of the pathogens *Bordetella* species and FCV are related risk factors.

Additionally, being housing in the shelter greater than 60 days and less than or equal to 180 days also appeared to be a related protective factor. When all significant factors were included in the fully adjusted model, the odds of FURD in a cat positive for *Bordetella* species was 3.143 (1.19, 8.32) times that of a negative cat. Overall, the detection of *Bordetella* species and FCV appeared to increase a cat's odds of FURD and being housed in the shelter for greater than 60 days and less than or equal to 180 days decreased the risk of FURD. The age of a cat and the detection status of *C. felis* were also found to confound the relationship between *Bordetella* species and the risk of FURD.

There were several limitations to this study. The rare detection of the pathogens *C. felis* and other *Mycoplasma* species in this population of cats and as such their detection was not significant. The inability to find all the variable information on shelter paperwork resulted in only 141 of the 176 shelter cats having a reported time in the shelter. While this limited the number of cats included in multivariate analysis, this also exclude the client owned cats from this analysis. There was limited data available and collected on treatments cats had received that may have affected detection of pathogens. Shelter protocols varied in treatment methods with common antimicrobials used including oral clindamycin, potentiated amoxicillin, oxytetracycline ointment, and oral doxycycline. The majority of these drugs have limited efficacy against both *C. felis* and *M. felis* but are effective against *Bordetella* species (Sparkes et al., 1999; Hartmann et al., 2008; Ruch-Gallie et al., 2008; Litster et al., 2012). Appropriate antimicrobial treatment could decrease the pathogen load before clinical signs abate and reduce the detection of bacterial pathogens. This did not lead to bias in the detection of viral pathogens.

Due to the nature of this cross-sectional study and the limited data available on the length of clinical signs prior to testing, cats were likely sampled during all stages of illness, recovery, and health. This sampling also affected the pathogen load, shedding, and detection. Additionally, animals were often maintained in isolation for a period after clinical signs have abated to ensure their health and protect the healthy adoption population, so these animals, while originally isolated for FURD signs, were also scored as asymptomatic at the time of sampling. It was also noted during this research that pure culture *Bordetella bronchiseptica* had significant denaturation during freeze thaw cycles and holding in refrigeration. This may have resulted in marked underestimates of *Bordetella* species prevalence in this cat population.

Conversely, this study utilized a population of cats in a unique geographical area and sampled from a large number of clinical healthy cats. The use of the large number of clinically healthy cats increases the power of pathogen association with disease. The results of this study suggest that the pathogens *Bordetella* species and FCV are important to the severity and demonstration of clinical signs of respiratory disease in cats and increase these signs. Older animals and cats housed in the shelter environment for 2-6 months have decreased disease severity. Overall, *Bordetella* species detection increased the odds of disease in cats by 3 times. All of these shelters also housed dogs and this may contribute to the presence of *Bordetella* species in the environment and possible transmission to the cat population.

It has been shown previously that cats housed in facilities with dogs have increased risk of developing FURD (Binns et al., 1999; Helps et al., 2005). Several

previous studies have demonstrated that younger cats have an increased risk of disease (Dinnage et al., 2003; Bannasch and Foley, 2005; Wong et al., 2013). This study also found that older cats have decreased odds of FURD which agrees with previous findings. This is likely due to these animals having been previously vaccinated or exposed to the pathogens and recovered. Additionally, older animals may be animals that have been housed in the shelter environment. Age was also found to be a confounder of the relationship between *Bordetella* species detection and FURD. This suggests that age may have an impact on whether a cat has *Bordetella* species present and detectable. In this study time in the shelter was also found to significantly affect the odds of a cat having FURD. Cats housed in the shelter for 2-6 months had a decreased risk of developing FURD in relation to those cats housed in the shelter for 1 month or less. One previous study suggested that exposure and disease occurred early in shelter stay for cats, sometime in the 6-12 day range, and that after that time FURD prevalence decreases with the majority of disease occurring within the first 50 days (Edwards et al., 2008). This study also agrees with this previous work.

FHV was detected in a large number of the cats in this study and was not found to be significantly related to FURD. This suggests that FHV and *M.felis* are endemic in the environment and animals are constantly exposed to these. The lack of association with disease in this study can be attributed to the long term exposure of the cats, early infection and maturity of the immune response, and neonatal immunity. It is important that both pathogens were detected at high levels suggesting that they are frequently shed

in the saliva and lacrimal fluids and contribute to the environmental pathogen load that naïve cats are exposed to on intake.

This study demonstrates that both *Bordetella* species and FCV are important pathogens in the development of FURD clinical signs in the studied shelter populations. Additionally, it appears that *C. felis* may not be a pathogen detected in cats with FURD very often and may not be contributing to FURD disease as commonly as other upper respiratory pathogens. Knowledge of the microbial make-up of the shelter population and of disease cats allows for triage of the population, and isolation and treatment of affected cats in a manner that is both effective and efficient. This study suggests that when triaging FURD in upper Midwestern shelters the main pathogens of concern should be *Bordetella* species and FCV. Additional research into the specific *Bordetella* species involved in FURD and infecting cats as well as effective measures to prevent the spread of these pathogens both within cat populations and to and from canine populations is necessary.

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GENERAL CONCLUSIONS

This research project was compilation of three studies to investigate FURD in Midwestern feline shelter populations and determine what pathogens and risk factors are present as well as validate a field deployable device capable of detecting pathogens for triage, biosecurity, and population management.

Results indicated that while the pathogens previously associated with FURD were present in these shelter populations (FHV, FCV, *C. felis*, *M. felis*, and *Bordetella* species), there are several other Mycoplasma species that can be detected in cats and may have involvement in the development and progression of FURD. The first study demonstrated detection of 12 Mycoplasma species previously unreported in cats. Of these 2 are known zoonotic species with transmission to and disease causation reported disease in humans. Overall, 15 Mycoplasma species were detected in cats in this study and 7 are known to be zoonotic species causing disease in humans. These discoveries greatly increase the knowledge about *Mycoplasma* host-specificity and suggest that cats can be carriers and potentially clinically affected by *Mycoplasma* species that were previously assumed to be host-specific pathogens. While detections of atypical Mycoplasma species were rare and could not be statistically correlated with disease, the presence of these pathogens in cats with severe FURD suggests that further investigation is warranted.

Validation of the field deployable point of need POCKIT™ device and Insulated Isothermal Polymerase Chain Reaction (iiPCR) for the common FURD pathogens illustrates a 5 stage pipeline for validation of these devices for field use and triage. The iiPCR was found to be sensitive, specific, and exclusive for the FURD pathogens in both

pure culture and in clinical samples and future evaluation of this technology will involve a field trial and development of standard operating procedures. The ability to have a fast, efficient, and cost-effective device and assays that can be implemented in a shelter environment for rapid pathogen detection allows for the triage, medical management, and population biosecurity necessary to prevent morbidity and mortality in shelter cats. Implementation of this technology in conjunction with vaccination, isolation, and other preventative medicine and biosecurity protocols can improve the general health of a shelters feline population which in turn increases the likelihood of adoptions and a healthy feline pet population.

The epidemiological study of this Midwestern feline population through a cross sectional study design allowed for the evaluation of pathogen prevalence in both clinically normal cats and those with FURD. Additionally, the factors time in the shelter, age, and sex were evaluated. This study evaluated a unique geographically location, incorporation of a high number of clinically normal cats, an utilized standardized molecular diagnostic testing to evaluate for pathogen presence. Overall, it was found that Midwestern shelter populations are very similar to those previously studied in that age and time in the shelter affect the feline health outcome. It was also found that the presence of the pathogens FCV and *Bordetella* species significantly increased the odds of disease for cats. *Bordetella* species are emerging infectious diseases in cats with an increasing number of studies recognizing their significant role in FURD in shelter populations. While work is still needed to differentiate the specific *Bordetella* species affecting these cats and determine if they have an identical genetic signature to *Bordetella* species infecting dogs, humans, and other domestic animal species, it is

important to recognize that control of *Bordetella* species is essential to management of FURD in felines.

Overall, the findings of these studies contribute to the body of knowledge regarding FURD in shelter housed cats and provide indications of areas where management and preventative medicine can implement disease preventing measures. Additionally, the future validation of a field deployable device and its translation into a shelter environment provides a tool for detection and triage of respiratory pathogens. All of these findings have direct translational abilities to improve the health of feline shelter populations.

APPENDIX A. LETTER OF INVITATION FOR SHELTER PARTICIPANTS

VCS, College of Veterinary Medicine
Iowa State University
Ames, IA 50011

March 31, 2014

(Shelter address here)

Dear (Veterinarian/Shelter director):

We would like to formally invite your shelter and its staff to participate in a sponsored summer investigatory project to be completed by veterinary students from Iowa State University in conjunction with clinicians and researchers from Iowa State. This project is sponsored by Maddie's Shelter Medicine Program, Morris Animal Foundation, and the Center for Advance Host Defenses and Immunobiotics and Translational Comparative Medicine. This project will involve sampling shelter cats and testing them for infectious agents which are known to cause feline upper respiratory disease and conjunctivitis.

During visits to your shelter I, and several other veterinary students under the supervision of veterinary clinicians, would like to sample your feline population over the summer. This sampling technique is the same one we have used in previous years and involves a short examination of cats (both health and overtly ill) as well as the collection of samples including swabs of the conjunctiva, nasal and deep-oropharynx. All sampling materials will be provided by the project. We will provide the results of the testing to shelter personnel so that this information may be used in individual treatment of feline URIs as well as overall shelter management for infectious disease control. When I put together the final report for this project shelters will not be individually identified. The culmination of this investigation will be a poster presentation this summer at the Summer Scholars poster day, as well as a poster presentation next summer at a Morris Animal Foundation convention.

We will be using the samples to develop the ability to test for disease causing agents, specifically Mycoplasma species, at ISU. Development of the ability to test at ISU will provide a rapid, alternative, and affordable test option for shelter personnel who deal with upper respiratory tract infections in cats. The final objective of this project is to provide shelters with not only better testing methods for infectious agents causing upper respiratory infections but also to aid in targeted management of infectious agents. This will allow for improved care and management of all, quicker recoveries, and less pain and suffering for animals, thus facilitating placement in homes faster.

We thank you in advance for consideration and participation in our study. Your participation is essential for us to attain our goals and advance feline infectious disease management in shelter settings. With your help we believe that we can conduct research that will not only benefit your shelter but improve the longevity and health of your feline adoptees lives. If any questions arise or you would like more detailed information on this project please do not hesitate to contact me, Uri Dornett at 513-290-0304 (email udornett@iastate.edu).

Sincerely,
Dr. Claudia J. Baldwin, DVM, MS
Uri Dornett, BS

APPENDIX B. CLIENT CONSENT FOR IN HOSPITAL SAMPLING OF LLOYD VETERINARY MEDICAL CENTER PATIENTS

Consent for Sample Collection and Testing for Infectious Diseases of the Eye and Upper Respiratory Tract

This Form is to be used in conjunction with ISU Veterinary Medical Center
"Consent to Treatment and/or Operation"

Title of Study: Epidemiological Study of Mycoplasma species in Felines Utilizing a Novel Pan-Myco PCR Assay

Investigators: Trujillo, Donnett, Jergens and Baldwin.

What is the purpose of this research study?

Historically, infectious ocular and upper respiratory diseases in cats has been attributed to the bacterial species Mycoplasma felis (as well as a second bacterium, Chlamydia, and the Feline Herpes Virus). More recently others have completed a small study suggesting that other species of Mycoplasma can be found in animals with clinical disease. We have developed a new molecular based detection assay to investigate the role of Mycoplasma felis and other Mycoplasma species in feline ocular and upper respiratory disease on a larger scale.

This study is a clinical research study and will be performed by a second year veterinary student, under the guidance of skilled clinical veterinarians and biomedical researchers. This student is interested in feline health, diagnosis, and treatment of infectious diseases. We thank you for your consideration in participating in this project

The purpose of this form is to inform you of the details of this study and to seek your consent for participation. Participation involves the testing of clinical specimens collected from your pet during the diagnostic workup in an attempt to determine the cause for your pet's illness.

What is involved in participation in the study?

We are asking that we collect several samples from your pet/cat for testing. Collection will be brief and non-invasive and will entail the collection of a swab of ocular fluid from both eyes, the nose, and one from the back of the mouth (oro-pharynx) by a trained veterinary technician, veterinary student or clinician. If at any time your pet becomes significantly stressed during the brief collection procedure, the collection will cease. Only one set of samples for this study is necessary; because we are collecting samples from cats with and without clinical signs of upper respiratory or ocular disease. Following testing, if we identify an infectious agent that may be causing disease in your cat this information will be released to your veterinarian, to potentially aid in the treatment of your pet at no cost.
Additionally a quick clinical scoring of your cats ocular and respiratory disease or lack there of will be documented.
.

What are the possible risks to my animal from being in this study?

During the collection of samples, if a pet struggles severely, there is minimal risk of ocular injury; this is why, if the animal struggles severely or develops difficulty breathing during the brief collection procedure, all sample collection will be stopped. In the event that ocular injury is sustained during sample collection, all costs of medical care related to the injury will be covered by the primary investigator.

In the event that your pet succumbs to its disease while in the hospital or at home, a post-mortem examination (animal necropsy) will be requested to determine the cause of death for the advancement of veterinary medicine and veterinary health research. The necropsy will be performed at no cost to you and with the utmost respect for your pet. Additionally your pet can be returned to you for burial. The need for a necropsy will be determined by the investigator and the ISU Attending Veterinarian and/or the Institutional Animal Care and Use Committee; the investigator will pay for any costs associated with the necropsy.

Will I have any costs from this research or will I be compensated?
No cost related to this study will be imposed on the pet owner. Results of the testing will be provided to the veterinarian free of charge.

Can my animal receive treatment if I do not want them to be in the research study?

Participation in this study is completely voluntary. You may choose not to have your animal participate and still receive veterinary care for its illness.

What measures will be taken to protect my privacy?

Data collected will be part of your pets medical record but will be shared with veterinarians associated with this project within the College of Veterinary Medicine at Iowa State University. Additionally, the veterinarian and veterinary researchers may use data generated from this study, including photographs and video images, in scientific/journal articles or presentations and for educational

Implemented: June 1, 2011

purposes. Neither you nor your animal will be identified individually in such articles, presentations, or educational programs, so the use of this data will be confidential.

Whom can I call if I have questions or problems?

You are encouraged to ask questions at any time during this study. For further information about the study, contact

Jessica Trujillo, DVM, PhD at 309-432-9468 trujill@illinois.edu

.....

OWNER SIGNATURE

Your signature indicates: (1) you voluntarily agree to your animal's participation in this study; (2) you are the legal owner of the animal or are an agent of the owner with authority to consent to the animal's participation in this study; and (3) you have read this Owner Consent Document and your questions have been satisfactorily answered. You will receive a copy of this Owner Consent Document prior to your animal's participation in the study.

Printed name of owner or agent: _____

Owner or Agent's Signature: _____

Name of Animal: _____

Date: _____

Witness (printed name): _____

Witness (signature): _____

Date: _____

VETERINARIAN/INVESTIGATOR STATEMENT

It is my opinion that the owner or owner's agent understands the purpose, risks, benefits, and the procedures that will be followed in this study and has voluntarily agreed to participate.

Veterinarian/Investigator Signature: _____

Date: _____

Testing Outcome: please list test performed and results (to be completed at a later date after sample testing)

PCR: _____

Other: _____

Print Clinicians Name: _____

APPENDIX C. FELINE RESPIRATORY DISEASE SCORE CARD

Shelter Myco Evaluation

SHELTER:	ROOM IN SHELTER:	DATE:	EVALUATOR:
CAT NAME:	MARKINGS:		TIME IN SHELTER:
AGE (RECORDS):	SOURCE: (SURRENDER, STRAY, ETC)		SEX:

GENERAL DEMEANOUR: 0-NORMAL 1-DEPRESSED 2-SEVERELY DEPRESSED

BCS: (1-9)	CRT: <2 >2	SKIN TENT (sec): <1 1-4 5-10 11-15 16-45	HYDRATION:

EYE EXAM

CONJUNCTIVITIS: Y N	KERATITS: Y N	UVEITIS: Y N

DISCHARGE: 0-NORMAL 1-CLEAR DISCHARGE 2-MUCOPURULENT DISCHARGE

SAMPLES TAKEN AND LABELED: LEFT EYE [ ] RIGHT EYE [ ]

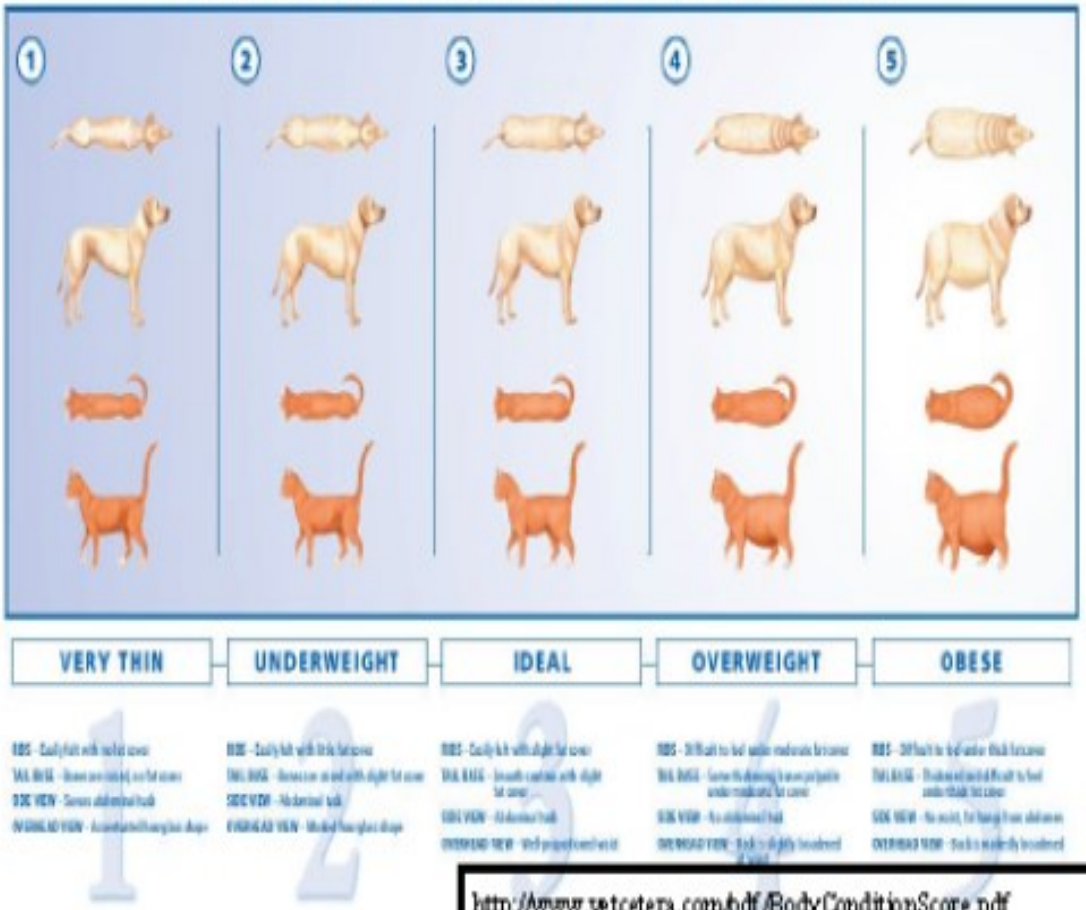
RESPIRATORY EXAM

LUNG SOUNDS: 0-NORMAL 1-CONGESTION OR DRY RALES 2-MOIST RALES 3-CONSOLIDATED AREAS	EFFORT: 0-NONE 1-MILD 2-MOD. 3-DYSPNEA	NASAL DISCHARGE: 0-NORMAL 1-CLEAR DISCHARGE 2-MUCOPURULENT	
		NOISE/SNEEZING: 0-NORMAL 1-OCCASIONAL 2-FREQUENT/SEVERE	
		GINGIVITIS: 0 1 2 3	PLAQUE: 0 1 2 3
		STOMITIS/FAUCITIS: 0 1 2 3	OTHER LESIONS:

0= NORMAL 1=MILD 2=MODERATE 3=SEVERE

SAMPLES TAKEN: NASAL [ ] ORO-PHARYNGEAL [ ]

OTHER NOTES/ANTIBIOTICS/TREATMENT REGIMEN:



http://www.vetcetera.com/pdf/BodyConditionScore.pdf

Swabs: use each swab one time

1. Right Eye (blue swab)
2. Left Eye (blue swab)
3. Nasal Plane (green swab) - if discharge present
4. Oro-pharyngeal (white swab)

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